DOCTOR OF PHILOSOPHY

Innovations in detecting food fraud using mass spectrometric platforms and chemometric modelling

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Award date:
2017

Awarding institution:
Queen's University Belfast

QUEEN'S UNIVERSITY BELFAST

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A thesis submitted to the

School of Biological Sciences,

Queens University Belfast

for the degree of

DOCTOR OF PHILOSOPHY

by

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September 2017

QUEEN'S UNIVERSITY BELFAST

Acknowledgements

First and foremost, I would like to thank my principal supervisor, Professor Chris Elliott for his excellent supervision throughout my time at the Institute for Global Food Security. His expertise, guidance and support throughout has been magnificent and made this thesis possible. I must also pay special thanks to Dr Olivier Chevallier for patiently teaching me how to operate the mass spectrometry instruments and sharing his expertise in analysing mass spectrometric data. His support throughout the project has enabled me to overcome many hurdles and challenges.

During the PhD, I have had the benefit of many colleagues who have been involved in my training in various scientific techniques used in this research. For this my thanks go to Brett Greer and Dr Simon Haughey within the Institute. Additionally, I would like to thank Dr Sara Stead, Dr Steven Pringle and Dr Julia Balog from the Waters Corporation for their patient help and support throughout this thesis.

I would like to thank the Biotechnology and Biological Sciences Research Council (BBSRC) and Waters Corporation for their funding, without which this research would not have been possible. Additionally, I would like to thank all those in the food industry in the UK and Ireland who supplied samples for me to analyse

Finally, I would like to pay my greatest thanks to my mum (Alison), dad (Clive), brother (Josh), sister (Maeve) and girlfriend (Tash) for their support, love and laughter throughout my research over the last three years.

Abstract

Food fraud is an economically motivated concept that has occurred within the food supply system since trading began. It has been defined as the deliberate and intentional substitution, addition, tampering or misrepresentation of food, food ingredients and food packaging for an economic gain. It is in many cases close to impossible for consumers and players in the food industry to identify. The aim of this thesis was to study whether mass spectrometric platforms, especially ambient mass spectrometry (AMS) techniques, coupled with chemometric modelling could play a prominent role in the detection of food fraud.

Firstly, a two-tier approach of Fourier-Transform infrared (FT-IR) spectroscopy and liquid chromatography-high resolution mass spectrometry (LC-HRMS) were used to analyse oregano samples and five potential adulterants (olive leaves, myrtle leaves, cistus leaves, sumac leaves and hazelnut leaves) thought to be used as bulking agents. LC-HRMS detected adulteration of oregano samples through biomarker identification which was achieved using chemometrics. Both analytical techniques were applied to seventy-eight commercially available samples obtained both within and outside the UK/Ireland. There was 100% agreement between the two tests which revealed that 24% of all samples analysed had some form of adulterants present with olive and myrtle leaves being the most commonly found adulterants.

Secondly, rapid evaporative ionisation mass spectrometry (REIMS) was used to determine the feasibility of fish species identification. Five white fish species (cod, coley, haddock, pollock and whiting) were analysed using an electrosurgical knife coupled to a quadrupole time-of-flight mass spectrometer (QTof). Principal component analysis (PCA) and linear discriminant analysis (LDA) models were generated exhibiting clear differences between the five species of fish. They were exported to a recognition software and used as a reference point allowing raw data from a sample unknown to the models to be assigned a species classification near-instantaneously (≈2s). A 98.99% correct classification of ninety-nine validation samples identified that REIMS is capable of both rapid and accurate results. Equally important, the

analysis of six suspected mislabelled ‘haddock’ samples were undertaken. Results from REIMS for all six samples was available within fifteen minutes whereas it took twenty-four hours using polymerase chain reaction (PCR), a genomic profiling technique commonly used for such studies.

In a further study, the REIMS technology was applied to four meat species (beef, goat, lamb and pork) to determine the quantitative abilities of the technology. As with the fish study, both PCA and LDA models showed clear differences between the four species. The models were exported to a recognition software to analyse adulterated beef burgers. Adulteration of beef burgers with goat was detectable at levels of 2% adulteration, whilst pork and lamb were detected at 5% and 10% respectively. However, the preparation of burgers made through a serial dilution process impacted the quantitative abilities of the REIMS technology with limits of detection (LOD) for each adulterant being higher compared to those not made through serial dilution. Chemometric analysis of the four-meat species did not result in the identification of unique species-specific markers. However, ions found to occur at more abundant levels in certain species were. They were identified as phospholipids with five different species being assigned; phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS).

Thus, within the PhD project the potential for ambient mass spectrometry to deliver very rapid and reliable detection of food fraud has been demonstrated.

Key words: Mass spectrometry; Chemometric modelling; Food fraud; Ambient mass spectrometry; Liquid chromatography-high resolution mass spectrometry; Fourier-Transform Infrared spectroscopy; Speciation, Adulteration; Authenticity.

Publications relating to this thesis

Black C, Chevallier OP, Elliott CT. The current and potential applications of Ambient Mass Spectrometry in detecting food fraud. *TrAC Trends in Analytical Chemistry*. 2016; 82:268-278.

Black C, Haughey SA, Chevallier OP, Galvin-King P, Elliott CT. A comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach. *Food Chem*. 2016; 210:551-557.

List of abbreviations

^{13}C-CYA	^{13}C Isotopically Labelled Cyanide
^{13}C-MEL	^{13}C Isotopically Labelled Melamine
5-HMF	5-Hydroxymethylfurfural
µA	Microamp(s)
µg	Microgram(s)
µL	Microlitre(s)
µm	Micrometre(s)
σ	Standard Deviation
A	Amp(s)
AMS	Ambient Mass Spectrometry
APCI	Atmospheric Pressure Chemical Ionisation
APGDDI	Atmospheric Pressure Glow Discharge Desorption Ionisation
ASAP	Atmospheric Solid Analysis Probe
ASAP-MS	Atmospheric Solid Analysis Probe-Mass Spectrometry
ASAP-TOF-MS	Atmospheric Solid Analysis Probe-Time of Flight Mass Spectrometry
ASTA	American Spice Trade Association
bp	Base-pair
BOLD	Barcode of Life Data Systems
C	Carbon
CFIA	Canadian Food Inspection Agency
$CHCl_3$	Chloroform
CI	Chemical Ionisation
cm	Centimetre(s)
COI	Cytochrome c Oxidase Subunit I gene
Da	Dalton
DAG	Diacylglycerol
DAPCI	Desorption Atmospheric Pressure Chemical Ionisation
DAPCI-MS	Desorption Atmospheric Pressure Chemical Ionisation-Mass Spectrometry
DAPPI	Desorption Atmospheric Pressure Photo Ionisation
DAPPI-MS	Desorption Atmospheric Pressure Photo Ionisation-Mass Spectrometry
DART	Direct Analysis in Real Time
DART(+)	Direct Analysis in Real Time Positive Ionisation Mode
DART(-)	Direct Analysis in Real Time Negative Ionisation Mode
DART-HRMS	Direct Analysis in Real Time-High Resolution Mass Spectrometry

DART-MS	Direct Analysis in Real Time-Mass Spectrometry
DART-QTof-MS	Direct Analysis in Real Time-Quadrupole Time-of-Flight-Mass Spectrometry
DART-TOF-MS	Direct Analysis in Real Time-Time of Flight Mass Spectrometry
DBDI	Dielectric Barrier Discharge Ionisation
DCBI	Desorption Corona Bean Ionisation
DEMI	Desorption Electrospray/Metastable-Induced Ionisation
DESI	Desorption Electrospray Ionisation
DESI-MS	Desorption Electrospray Ionisation-Mass Spectrometry
DESSI/(EASI)	Desorption Sonic-Spray Ionisation / Easy Ambient Sonic-Spray Ionisation
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
EADSI	Electrode-Assisted Desorption Electrospray Ionisation
EASI-MS	Easy Ambient Sonic-Spray Ionisation-Mass Spectrometry
EC	Electron Capture
EESI	Extractive Electrospray Ionisation
EFSA	European Food Safety Authority
EI	Electron Ionisation
ELDI	Electrospray-Assisted Laser Desorption Ionisation
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	Economically Motivated Adulteration
ESA	European Spice Association
ESI	Electrospray Ionisation
ESTASI	Electrostatic Spray Ionisation
EU	European Union
eV	Electronvolt(s)
FA	Fatty Acid(s)
FAO	Food and Agriculture Organisation of the United Nations
FFA	Free Fatty Acid(s)
FSA	Foods Standard Agency
FT-ICR	Fourier Transform Ion Cyclotron Resonance
FT-IR	Fourier Transform Infrared
FWHM	Full Width at Half Maximum
g	gram(s)
GC	Gas Chromatography

GC-MS	Gas Chromatography-Mass Spectrometry
GDP	Gross Domestic Product
GMA	Grocery Manufacturers Association
GVA	Gross Value Added
H	Hydrogen
H_2O	Water
HALDI	High-Voltage-Assisted Laser Desorption Ionisation
HALDI-MS	High-Voltage-Assisted Laser Desorption Ionisation-Mass Spectrometry
HAPGDI	Helium Atmospheric Pressure Glow Discharge Ionisation
HCA	Hierarchical Cluster Analysis
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Pressure Liquid Chromatography
HPLC-MS	High Pressure Liquid Chromatography-Mass Spectrometry
HRAM	High-Resolution Accurate-Mass
HRMS	High Resolution Mass Spectrometry
ICO	International Coffee Organisation
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IE	Ionisation Energy
IGD	Institute of Grocery Distribution
IMS	Ion Mobility Spectrometry
IPA	Isopropanol
IR	Infrared
IR-LAMICI	Infrared-Laser Ablation Metastable-Induced Chemical Ionisation
IRMS	Isotope Ratio Mass Spectrometry
JeDI	Jet Desorption Electrospray Ionisation
kg	Kilogram(s)
kV	Kilovolt(s)
LAESI	Laser Ablation Electrospray Ionisation
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LDA	Linear Discriminant Analysis
LDSPI	Laser Desorption Spray Post-Ionisation
LDSPI-MS	Laser Desorption Spray Post-Ionisation-Mass Spectrometry
LESA	Liquid Extraction Surface Analysis

LESA-MS	Liquid Extraction Surface Analysis-Mass Spectrometry
LeuEnk	Leucine Enkephalin
LOD	Limits of Detection
LOQ	Limits of Quantitation
LS-APGD	Liquid Sampling-Atmospheric Pressure Glow Discharge
LSI	Laser Spray Ionisation
LTP	Low Temperature Plasma
LTP-MS	Low Temperature Plasma-Mass Spectrometry
mAbs	Monoclonal Antibodies
MALDESI	Matrix Assisted Laser Desorption Electrospray Ionisation
MALDI	Matrix Assisted Laser Desorption/Ionisation
MEL-d_6	Deuterated Melamine
MeOH	Methanol
mg	Milligram(s)
MICI	Metastable-Induced Chemical Ionisation
min(s)	Minute(s)
MIPDI	Microwave Induced Plasma Desorption Ionisation
MIR	Mid-Infrared
MIR-ATR	Mid-Infrared Attenuated Total Reflectance
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
MPa	Megapascal
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MTBE	Methyl-Tert-Butyl Ether
m/z	mass/charge ratio
N	Nitrogen
Na	Sodium
ND:YAG	Neodymium-Doped Yttrium Aluminium Garnet
ng	Nanogram(s)
NH_4	Ammonium
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
^oC	Degrees Celsius
OOO	Trioleoyl-glycerol

OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PA	Phosphatidic Acid(s)
PA	Proton Affinity
PADI	Plasma Assisted Desorption Ionisation
PC	Phosphatidylcholine(s)
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDO	Protected Designation of Origin
PE	Phosphatidylethanolamine(s)
PGI	Protected Geographical Indication
PhEur	European Pharmacopoeia
PI	Penning Ionisation
PI	Phosphatidylinositol(s)
ppm	Parts Per Million
PRESS	Predicted Residual Sum of Squares
PS	Paper Spray
PS	Phosphatidylserine(s)
PS-MS	Paper Spray-Mass Spectrometry
PT	Proton Transfer
PTR-MS	Proton Transfer Reaction-Mass Spectrometry
QC	Quality Control
QTof	Quadrupole Time-Of-Flight
RAPD	Random Amplified Polymorphic DNA
REIMS	Rapid Evaporative Ionisation Mass Spectrometry
RMSECV	Root Mean Squared Error of Cross Validation
RPLC	Reversed-Phase Liquid Chromatography
s	Second(s)
SCARs	Sequence-Characterised Amplified Region Markers
SFC-MS	Supercritical Fluid Chromatography-Mass Spectrometry
SIMCA	Soft Independent Modelling of Class Analogy
SNV	Standard Normal Variate Technique
SSI	Sonic-Spray Ionisation
TAG	Triacylglycerol(s)
TG	Triglyceride(s)
TD-NMR	Time Domain-Nuclear Magnetic Resonance
TI	Thermal Imprinting

TIC	Total Ion Count
TI-EASI-MS	Thermal Imprinting-Easy Ambient Sonic-Spray Ionisation-Mass Spectrometry
TM-DESI	Transmission Mode Desorption Electrospray Ionisation
TQD	Triple Quadrupole
TSG	Traditional Speciality Guaranteed
UK	United Kingdom
UPLC-QTof-MS	Ultra-Performance Liquid Chromatography-Quadruple Time of Flight Mass Spectrometry
USA	United States of America
UV	Ultraviolet-Visible
V	Volt(s)
VIP	Variable Importance in Projection
v/v	Volume/Volume ratio
W	Watt(s)
WHO	World Health Organisation
WLR	Within Laboratory Reproducibility
w/w	Weight/Weight ratio

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1. Introduction

Black C, Chevallier OP, Elliott CT. The current and potential applications of Ambient Mass Spectrometry in detecting food fraud. *TrAC Trends in Analytical Chemistry*. 2016; 82:268-278.

1. Food fraud

With a growing global human population and longer life expectancies, the increased demand for food has led to the rapid growth of the food industry. In 2013 the agri-food sector contributed £103 billion to the United Kingdom (UK) economy, which accounted for 7.6% national Gross Value Added (GVA).[1] GVA measures the contribution of each producer, industry or sector in the UK and is used to estimate Gross Domestic Product (GDP) and so the economic state of the whole economy. Total consumer expenditure on food, drink and catering in the UK is worth £196 billion, a rise of 4% compared with 2012. More recently, the Institute of Grocery Distribution (IGD) estimated that the UK food retail industry has a turnover of £177.5 billion in the year for May 2015, with projections for £200 billion of sales in 2020.[2] Horizon forecasts that the UK foodservice market is worth £46.6 billion in 2014 and that this will rise to £56.3 billion in 2019.[3] On a global scale the IGD expects the value of the world's grocery market to increase by a third between 2015-2020 reaching $11.8 trillion in 2020, with the greatest contribution in growth being driven by lower-middle income countries such as India, Indonesia and Nigeria.[4] Table 1 identifies how this value was established, showing the grocery market size forecasts for the major international markets between 2015-2020 in US dollars (billions).[5]

The maximisation of profits is the primary target for any company. However, within the food industry, where many businesses are profitable and this profit is made by working within legal frameworks, there are some cases where increased profit margins are made illegally through the sale of fraudulent food.

1.1 Aspects of food fraud

Food fraud is an economically motivated concept that has occurred within the food production and retail sectors since trading began and is defined as the deliberate and intentional substitution, addition, tampering or misrepresentation of food, food ingredients and food packaging for an economic gain.[6,7]

US dollars (billons)	2015	2016	2017	2018	2019	2020
UK	310	320	328	336	344	352
United States of America (USA)	1,078	1,122	1,169	1,216	1,260	1,305
China	1,120	1,174	1,237	1,314	1,400	1,491
India	503	566	635	713	802	901
Japan	457	464	469	474	479	485
European Union (EU)	1,787	1,829	1,872	1,918	1,970	2,024
North America	1,186	1,234	1,286	1,337	1,385	1,434
Asia	3,034	3,240	3,466	3,724	4,012	4,325
Total world	8,757	9,302	9,861	10,464	11,114	11,814

Table 1. Institute of Grocery Distribution (IGD) grocery market size forecasts between 2015-2020 for international markets (US dollars-billions).[5]

The Grocery Manufacturers Association (GMA) of America estimates that food fraud costs the global food industry between $10 billion and $15 billion per year and that it affects up to 10% of all the food that is eaten in the developed world and 20% in the developing world.[8] Food fraud can be broken down into two groups:

1. The sale of food which is unfit or potentially harmful to the consumer. This includes food that is sold past its designated sell by date or with an unknown geographic origin.
2. Deliberate misrepresentation of food. This involves the substitution/addition of a product with a cheaper alternative, which has been known in the beef and fish industries; or adding dangerous substances such as dyes to spices. False statements regarding the source of ingredients would also fall under this category along with the sale of meat from animals that are stolen or illegally slaughtered.

To combat this ever-growing problem, many international food standards and regulations have been introduced. The EU food labelling directive 2000/13 article 2 requires that consumers

must not be misled regarding the characteristics of food, in particular the nature, identity, manufacture, origin and quality.[6]

Economically motivated adulteration (EMA) of food often goes undetected until it is too late to rectify the issue and therefore, it poses a substantial health risk.[9] Such is the anxiety now that the World Health Organization (WHO) stated that food contamination, whether it be deliberate or accidental, is one of the major public health threats of the 21st century.[10] The impact that food adulteration can have on the public's health very much depends on what adulterant is used and the extent of any contamination. The public's health can be put at an immediate risk with the inclusion of toxic or lethal contaminants, which is known as direct food fraud. Examples of this include the melamine scandal and the substitution of olive oil with poorly refined peanut oil. The harmful effects of food fraud may require a long-time exposure to the adulterant such as the addition of the illegal Sudan dyes to spices.[11] This phenomenon has been described as indirect food fraud.[7]

The concept of food fraud can be broken down into seven formats; tampering, theft, over-run, diversion, adulteration, counterfeit and simulation.[7] Recent food scandals have identified that food adulteration/authenticity is an operation which is becoming increasingly more routine, with the common theme behind all being the huge profits that can be procured by food gangsters and criminals. The adulteration of food is an economically motivated principle which can be defined as a component of the finished product being adulterated. There are many different manners in which this act can be carried out, but the three most common attempts at it are; substitution, addition and geographic fraud.

1.1.1 Substitution

Substitution is the complete or partial replacement of a food ingredient with a less expensive replacement. This would normally involve the addition, dilution or extension of an authentic ingredient with an adulterant.[12] Two global examples of this were the 2008 Chinese milk scandal and the 2013 European horsemeat scandal. The 2008 Chinese milk scandal involved the adulteration of milk and infant formula with melamine. It was reported that there were 300,000 victims with six children dying from kidney stones and other kidney related injuries.[13]

The price of all commodities is dictated by the basic economic principle of supply and demand. However, the price of milk is also dictated by their protein content, which is measured by the amount of nitrogen present. Melamine has a high nitrogen content containing 67% nitrogen by mass. As a result, melamine was added to milk to increase the nitrogen content and therefore, increase the price of the product. The scandal sent shock waves through the Chinese food industry as well as government departments and led to Chinese manufacturers sourcing their products abroad, most notably New Zealand, as the Chinese public had no confidence in their domestic dairy products.

The 2013 European horse meat scandal involved the adulteration of meat products with the non-declared species that was horsemeat.[14] Unlike the Chinese milk scandal, the horse meat scandal did not pose the same level of health risks to the public. Nevertheless, the principles of the two scandals were the same, with companies and intermediaries attempting to maximise profits through the sale of fraudulent food and drink. The UK Foods Standard Agency (FSA) found that beef products had contained horsemeat, which led to a large scale recall by many UK retailers.[15] Because of this scandal, the Elliot Report was produced to investigate the integrity and assurance of food supply networks within the United Kingdom.[16]

The implications of the European horsemeat scandal are still not fully known. However, in the May 2015 edition of The Grocer, the EU agency Eurojust stated that authorities in France, Belgium, Germany, Ireland, Luxembourg, the Netherlands and the UK had raided dozens of commercial and private outlets as part of an ongoing investigation into an organised criminal network involved in trading illegal horsemeat.[17] It was believed that the gang were slaughtering thousands of horses that were unfit for human consumption, but it was unclear as to whether or not the actions carried out by the gang were related to the horsemeat scandal in 2013.

1.1.2 Addition

Addition is when small amounts of a lower quality ingredient are added to foodstuffs to boost margins. The highest profile case of this in recent times was the addition of Sudan dyes to spices. Sudan dyes are phenyl-azoic derivatives and have been used as colouring materials for

plastics and shoes.[18] However, their degradation products are potential carcinogens and teratogens and therefore, pose health risks. The price of spices is normally dictated by the intensity of their vibrant colours and therefore, dyes are added to enhance the intensity and thus, increase the price.[19] In 2005 the UK experienced an extensive rapid alert action. It was found that products contacting chilli powder such as Worcester sauce, pizzas and pot noodles contained Sudan contaminants. Therefore, all products were subjected to complete recalls. Since then the EU Commission Decision 2005/402/EC requires that all chilli-, curry-, and curcuma-containing food products and palm oil coming into any EU member state are certified to be free of Sudan dyes.[18,20] Other illegal carcinogenic dyes such as basic red 46, which is analogous to Sudan dyes, have also been detected in spices such as sumac spice.[21]

1.1.3 Geographic fraud

Moving from food safety to frauding standards, the EU introduced three schemes to ensure authentic geographic labelling of food products. These were protected designation of origin (PDO), protected geographical indication (PGI) and traditional speciality guaranteed (TSG).[22] In 2011 there were 1029 quality foods registered within the EU of which 518 were PDO, 472 PGI and 39 TSG, with Italy having the highest number of recognitions with 232 food products.[23] In the UK, examples include Stilton Cheese (PDO), Cornish clotted cream (PDO), Jersey Royal Potatoes (PDO), Scottish wild salmon (PGI) and Traditional Farm Fresh Turkey (TSG).[24]

Water buffalo mozzarella is an Italian cheese recognised as a PDO product. It must be produced, processed and prepared within Central-Southern Italy including Campania, Lazio, Puglia and Molise regions.[25] Fresh buffalo milk must also be used in the manufacturing of the cheese for it to be qualified under the 'Mozzarella di Bufala Campana' trademark. However, common adulteration of this cheese is the addition of cheaper cow milk to the buffalo milk. Selling cheese that has the 'Mozzarella di Bufala Campana' trademark with cow milk is geographic fraud and deceiving the consumer.

The adulteration and fraudulent sale of food is growing at a rapidly rising rate, with all foods susceptible to the process. Certified labels such as 'Organic' and 'Fair Trade' goods may also

be affected by food fraudsters, with Europol indicating in the May 2015 edition of The Grocer that along with fake organic goods, which are already a growing problem in the food industry, Fair Trade fakes could be the next fraud scandal.[26] Additionally, Europol also indicated that Mediterranean countries such as Egypt and Turkey were responsible for a large share of counterfeit products within the food and drink industry coming into the EU.

1.2 The detection of food fraud

Food fraud has led to the public having little faith in the authenticity of the food that they are purchasing. Consumers, authorities and the reputable food industry are now demanding greater controls on the quality of food, the authenticity and traceability of food and general food safety. Reviews carried out by *Ellis et al.*, *Reid et al.*, *Reinholds et al.* and *Castro-Puyana et al.* signify the amount of work that has been dedicated in detecting the adulteration, authenticity, traceability, safety and quality of food. Methods of detection that have been utilised include; spectroscopic techniques such as ultraviolet-visible (UV), mid-infrared (MIR), mid-infrared attenuated total reflectance (MIR-ATR) near infrared (NIR), Fourier transform infrared (FT-IR), Raman, fluorescent; nuclear magnetic resonance (NMR); isotope ratio mass spectrometry (IRMS); inductively coupled plasma mass spectrometry (ICP-MS); proton transfer reaction-mass spectrometry (PTR-MS); high performance liquid chromatography (HPLC) and gas chromatography (GC); mass spectrometry (MS) techniques coupled with chromatography such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS); electronic nose; deoxyribonucleic acid (DNA) based technologies such as polymerase chain reaction (PCR); immunological technologies such as enzyme-linked immunosorbent assay (ELISA) and thermal techniques such as differential scanning calorimetry (DSC).[27-30] However, most of these techniques require long and complex sample preparation and assay times. Ambient mass spectrometry (AMS) is a relatively new field of analytical chemistry which has the potential overcome these issues, whilst giving results that are comparable with other conventional techniques.

2. Ambient mass spectrometry (AMS)

LC-MS has long been utilized to investigate metabolic profiling of animal, human and plant tissues.[31,32] Ionisation techniques such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have worked very well in separating analytes from a solution-phase matrix at atmospheric pressure and transferring free ions into a vacuum environment for MS analysis.[33] However, an issue with all atmospheric pressure ionisation sources is the long, often complex and expensive sample preparation time.

2.1 The creation of ambient mass spectrometry (AMS)

AMS was first identified in 1998 when Fenn, in his patent, anticipated paper spray mass spectrometry (PS-MS) when describing a direct ionisation method employing cellulose based materials.[34] However, the first published work by *Wang et al.* using PS-MS did not occur until 2010 and as a result, desorption electrospray ionisation (DESI) is widely regarded as the first ambient ionisation technique to be created in 2004 by *Takats et al.*[35,36] Their new ionisation technique allowed samples to be analysed direct and rapidly in the open air, with no sample preparation required.[37] *Takats et al.* initially stated that DESI was capable of analysing proteins and protein complexes, carbohydrates, oligonucleotides, industrial polymers and small organic molecules.[38] The research group observed that the protein DESI spectra were identical to that of ESI spectra, establishing that the results obtained from the DESI source were comparable with that to conventional techniques such as LC-MS. In 2005, two more AMS techniques were published; *Cody et al.* introduced direct analysis in real time (DART) and *McEwen et al.* created the atmospheric pressure solid analysis probe (ASAP).[39,40] Table 2 identifies the present applications of the three ambient ionisation techniques, which range across various industries including pharmaceuticals, forensics and chemical warfare.

Ambient ionisation technique	Applications	References
Desorption electrospray ionisation (DESI)	Forensics, public safety, explosives, toxic industrial compounds, chemical warfare agents, pharmaceuticals, industrial polymers, small organic molecules, proteins, oligonucleotides, carbohydrates and food analysis.	36,38,41
Direct in real time analysis (DART)	Chemical warfare agents, pharmaceuticals, metabolites, peptides, oligosaccharides, synthetic organics, organometallics, drugs of abuse, explosives for forensics and security, toxic industrial chemicals, food analysis and medicinal analysis	39
Atmospheric solid analysis probe (ASAP)	Pharmaceuticals, drugs, nucleosides, polymers, coal-related model compounds, steroids and food analysis	40,42,43

Table 2. Applications of desorption electrospray ionisation (DESI), direct in real time analysis (DART) and the atmospheric analysis probe (ASAP).

2.2 The mechanisms and evolution of ambient mass spectrometry (AMS)

Further development of ambient ionisation techniques has been undertaken to the point now where there are over thirty different techniques available.[44] Ambient ionisation techniques can be classified into three groups based upon their different ionisation mechanisms; (1) Spray or jet ionisation such as DESI where charged droplets are produced from an electrospray needle at a high voltage; (2) Electric discharge ambient ionisation, such as DART where ions, electrons and metastable atoms are produced using helium/nitrogen and a corona discharge; (3) An ambient gas-, heat- or laser assisted desorption/ionisation technique such as ASAP where a solid or liquid sample is ionised at atmospheric pressure between (300^{o}C-500^{o}C).[45]

Spray or jet ionisation	Electric discharge ambient ionisation	Ambient gas-, heat- or laser assisted desorption/ionisation
Desorption atmospheric pressure photo ionisation (DAPPI)	Atmospheric pressure glow discharge desorption ionisation (APGDDI)	Atmospheric solid analysis probe (ASAP)
Desorption electrospray/metastable-induced ionisation (DEMI)*	Desorption atmospheric pressure chemical ionisation (DAPCI)	Extractive electrospray ionisation (EESI)
Desorption electrospray ionisation (DESI)	Direct analysis in real time (DART)	Electrospray-assisted laser desorption ionisation (ELDI)
Desorption sonic-spray ionisation (DESSI) / Easy ambient sonic-spray ionisation (EASI)	Dielectric barrier discharge ionisation (DBDI)	High –voltage-assisted laser desorption ionisation (HALDI)
Electrode-assisted desorption electrospray ionisation (EADSI)	Desorption corona beam ionisation (DCBI)	Infrared-laser ablation metastable-induced chemical ionisation (IR-LAMICI) *
Electrostatic spray ionisation (ESTASI)	Helium atmospheric pressure glow discharge ionisation (HAPGDI)	Laser ablation electrospray ionisation (LAESI)
Jet desorption electrospray ionisation (JeDI)	Liquid sampling-atmospheric pressure glow discharge (LS-APGD)	Laser desorption spray post-ionisation (LDSPI)
Liquid extraction surface analysis (LESA)	Low temperature plasma (LTP)	Laser spray ionisation (LSI)
Paper spray (PS)	Microwave induced plasma desorption ionisation (MIPDI)	Matrix assisted laser desorption electrospray ionisation (MALDESI)
Transmission mode desorption electrospray ionisation (TM-DESI)	Plasma assisted desorption ionisation (PADI)	Rapid evaporative ionisation mass spectrometry (REIMS)

*indicates that both DEMI and IR-LAMICI ionisation mechanisms have traits similar to that of an electric discharge ambient ionisation mechanism and can therefore be grouped in two different ionisation mechanism classes.

Table 3. The grouping of ambient ionisation techniques based upon their ionisation mechanisms

Table 3 outlines which ambient ionisation techniques are characteristic of the three mechanisms described previously. Under the mechanism of spray or jet ionisation is a technique known as desorption electrospray/metastable-induced ionisation (DEMI). This technique, according to *Nyadong et al.* integrates the benefits and circumvents the limitations of both DESI and (DART)-type metastable-induced chemical ionisation (MICI).[46] Thus, it can be operated in three different ionisation modes; (i) a spray or jet ionisation: DESI; (ii) a metastable–induced chemical ionisation (MICI): DART; (iii) a multi-mode: DEMI.[46] Although table 3 has DEMI situated under the ionisation mechanism of spray or jet ionisation, theoretically it can also reside under electric discharge ambient ionisation. Operating DEMI in multi-mode allows a wider range of molecules or analytes to be analysed as DESI is suited towards the analysis of high molecular weight, polar and non-volatile molecules whereas DART is more suited towards the analysis of low molecular weight and low polarity molecules. Additionally, infrared laser ablation metastable-induced chemical ionisation (IR-LAMICI) is also characteristic of two of the ionisation mechanisms, as described by *Galhena et al.* when they stated that IR-LAMICI integrates both an infrared (IR) laser ablation and direct analysis in real time (DART)-type metastable-induced chemical ionisation.[47] Firstly, IR laser pulses impinge the sample surface ablating surface material and then a portion of ablated material reacts with the metastable reactive plume facilitating gas-phase chemical ionisation of analyte molecules generating protonated or deprotonated species in positive and negative ion modes, respectively.[47]

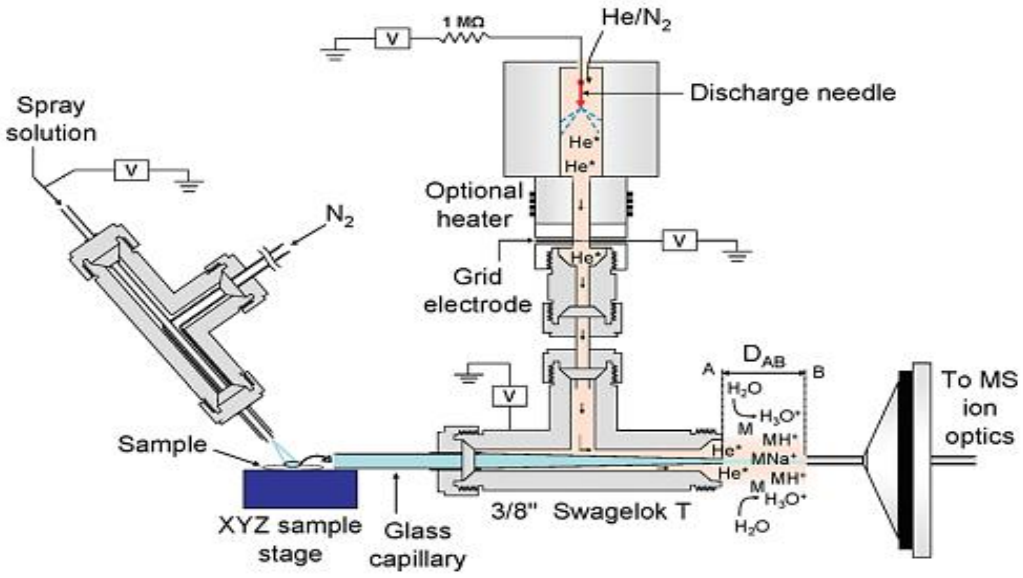


Figure 1. Schematic of the multi-mode DEMI mechanism taken from the work undertaken by *Nyadong et al.*[46] The higher polarity molecules are ionised by the DESI source (left) whilst the lower polarity molecules are ionised by the DART source (middle). The DEMI mechanism can be operated utilising both ionisation modes together or each one separately.

3. The analysis of food using ambient mass spectrometry

Food and drink commodities	Issue(s) addressed/analysed using ambient mass spectrometry	Ambient mass spectrometry (AMS) techniques	Issue(s) addressed/analysed using conventional techniques	Conventional techniques	References
Meat	Meat speciation/authentication; chicken feed control; Triacylglycerol (TAG), diacylglycerol (DAG) and free fatty acid (FFA) profiles of dry-cured ham	DART-MS, LESA-MS, DESI-MS, PS-MS, EASI-MS	Meat authentication/adulteration; mycotoxins in chicken feed	Stable ratio analysis, PCR, ELISA, NIRS, Raman, LC-MS, MIR-ATR	48-62
Fish	Dietary supplementation; geographic profiling of dried sea cucumber; lipidomic profiling of caviar; analysis of salmon, trout and sardine	DART-MS, DAPCI-MS, EASI-MS	Frozen/fresh differentiation; fish authentication/ mislabelling; mycotoxins in fish feed	Raman, PCR-ELISA, FT-(N)IR, LC-MS, NMR, GC-MS	63-72
Milk	Identification of melamine, dicyandiamide and cyanuric acid in milk powder, liquid milk, condense milk and soy milk; animal species origin	DAPCI-MS, DESI-MS, DART-MS, LTP-MS	Milk authenticity; animal species origin; adulteration of soy milk and yak milk	TD-NMR, MIR, PCR, NIRS, ELISA	73-83
Dairy products	Butter cholesterol levels; cheese adulteration with plant oils; analysis of margarine	DAPPI-MS, DESI-MS, DART-MS	Cheese adulteration; butter adulteration	LC-MS, NMR	25,75,84-86
Herbs, spices and sauces	Addition of illegal dyes; addition of additives; geographic discrimination of star anise; cinnamon authentication.	DAPCI-MS, DESI-MS, ASAP-MS, DART-MS, PS-MS	Contaminant analysis and adulteration in herbs and spices. Pesticides in herbs	NMR, ICP-MS, UV/Vis, NIRS, Raman, FT-IR LC-MS, GC-MS	19,29,43,49,87-94
Oils, nuts and condiments	Olive oil adulteration; geographic profiling of olive oil; quantitative analysis of 5-hydroxymethylfurfural in honey; fingerprinting of yogurt	DART-MS, EASI-MS, LDSPI-MS, PS-MS	Adulteration of olive and argon oils; analysis of balsamic vinegar; authenticity of hazelnuts	GC-MS, electronic nose, NMR, NIRS	95-102
Cereals	Mycotoxins and pesticides in cereals	DART-MS	Mycotoxins in wheat; herbicides in maize; pesticides in corn, oat, rice and wheat	LC-MS/MS, GC-MS, ELISA	103-106
Fruit and vegetables	Pesticides in fruit and vegetables, differentiation of organically and conventionally grown peppers and tomatoes	LTP-MS, PS-MS, LC/DBDI-MS, DART-MS	Identification of animals in vegetarian food; metabolic profiling of fruit; pesticides in fruit	PCR, NMR, LC-MS	107-113
Drinks	Recognition of beer brands; fungicides in wine; analysis of cola; origin and post-harvest methods of coffee beans; analysis of sports drinks	DART-MS, LTP-MS, PS-MS, EASI-MS, DESI-MS	Brandy adulteration, wine adulteration; authenticity of whiskey; ground coffee adulteration; pesticides in tea	Fluorescence spectroscopy, stable isotope ratio, IRMS, electronic nose, GC/MS, NIRS, MIRS, LC-MS	49,114-124

Table 4. The issues within food analysis that have been addressed by ambient mass spectrometry and conventional techniques since 2009.

Most, if not all the food commodities that appear on the shelves of supermarkets are either susceptible or have already been exposed to some form of food fraud. With greater controls and tests being demanded by the authorities and food industry, analytical techniques already play a key role in detecting the adulteration of food. Table 4 summarises the issues that have been addressed within several different food commodities using conventional techniques. Additionally, the table also outlines which of these issues have or have not been assessed using AMS. However, what this table does not address is the ability or indeed inability of AMS techniques to detect the adulteration in a fit for purpose manner. AMS continues to evolve and some of the techniques have excelled and been proven to produce accurate and reproducible results, whilst others have fallen short. This review attempts to identify the most recent work carried out using AMS, providing various scenarios where the technique(s) have worked very well, the technique(s) which have shown indications of their potential and others where the technique(s) have not produced data of much promise.

3.1 Desorption electrospray ionisation (DESI)

DESI is an imaging technique which is principally based on passing charged droplets through an electrospray needle at a high voltage, which are pneumatically assisted with nitrogen gas. The gas flow provides the charged droplets enough kinetic energy to hit the sample surface, even if an electrostatic charge is residing on the sample surface.[125] The droplets come in at an angle α relative to the sample surface, hit the desorbed analyte molecules to produce desorbed micro droplets which are then electrostatically repelled towards the MS inlet at angle β. The ionisation process depends very much on the size of the molecules being analysed. For biological macromolecular molecules, such as proteins and peptides the process is referred to as droplet pickup. The sample surface is pre-wetted by the initial charged droplets to form a micron thickness solvent film where analytes are dissolved. These are then impacted by the latter charged droplets to produce more droplets, which then become singly and multiply charged through electrospray processes.[37]

For low molecular weight and nonpolar molecules, the ionisation process is thought to involve charge transfer. This process can happen in three ways depending on the set up of the equipment and the properties of the sample being analysed:

1. Charge transfer between the solvent ion and analyte on the surface.
2. Charge transfer between a gas phase ion and analyte on the surface, which occurs when the charged droplets evaporate before reaching the samples surface due to a large distance between the spray and sample.
3. Charge transfer between a gas phase ion and a gas phase analyte molecule, which occurs when the sample has a high vapour pressure.

The quality of desorption electrospray ionisation mass spectrometry (DESI-MS) spectra are determined by certain parameters such as; the velocity at which the charged molecules hit the sample surface, the angle at which the charged molecules collide with the surface and the angle at which the desorbed micro droplets repel from the sample surface towards the MS inlet. These geometric parameters can both be key and detrimental to the quality of the DESI spectra. If not optimised it can result in limited sensitivity and/or selectivity. Sample surface properties such as hardness and shape may also influence spectral quality as well as the flow rate of the solvent and voltage of the electrospray needle.

3.1.1 The analysis of food using desorption electrospray ionisation mass spectrometry (DESI-MS)

DESI-MS has mostly been applied to quality control, pharmaceutical and forensic analysis due to its ability to screen samples directly and rapidly and analyse specimens in different forms (tablets, gels etc.).[41,126] Compared to conventional LC-MS, the literature indicates very little research has been undertaken regarding the detection of food adulteration using DESI-MS. Various issues have been addressed with regards to the analysis of food including the analysis of lipids in butter products, the identification of triglycerides (TG) in edible oils and margarine and the differentiation of post-harvest methods of coffee beans.[84,85,119] However, what work that has been carried out using DESI-MS has indicated that it is not particularly

effective at detecting food adulteration compared to other AMS techniques, with the lack of chromatographic separation being cited as the main issue.

Yang et al. attempted to detect the adulteration of milk using DAPCI-MS but they also investigated how DESI-MS could be used and how the results compared.[73] DAPCI is a variant of DESI and involves the use of a corona-discharge ion source to produce ions instead of an ESI sprayer.[38] Whereas the ionisation mechanism of DESI is characteristic of ESI, the ionisation mechanism of DAPCI is more akin to that of APCI. The literature suggests that DAPCI-MS is potentially more suited to detecting the adulteration of food compared to DESI-MS, as *Chen et al.* showed when investigating the adulteration of tomato sauce with Sudan dyes.[87] At the outset of their study, *Yang et al.* gathered a DAPCI-MS spectrum of authentic melamine (10ng) and found the mass ion at m/z 127 and the fragment ions at m/z 110, 85 and 60. They then tested powdered milk samples contaminated with melamine and found both the parent and daughter ions of this compound. However, when they utilised DESI-MS on a 10 µL milk sample containing 10 ppm melamine, the melamine daughter ions were not picked up and instead ions of m/z 109, 84 and 81 were detected. It was concluded that DESI-MS could not be used to detect melamine contamination at a meaningful concentration. It is important to note that *Yang et al.* found that by drying the liquid milk samples at 120^{o}C, they could detect weak melamine signals with the correct daughter ions using DESI-MS. Although, having undertaken this research, they still stated that powdered milk should not be directly analysed by an open-air DESI source because the fine particles started to contaminate the source region when the gas pressure was higher than 0.2 MPa.

A summary of the work using DESI-MS, with particular reference to food analysis ranging from the addition of Sudan dyes in tomatoes and spices, to the addition of sweeteners and food forensics, including the work undertaken by *Yang et al.* was carried out by *Nielen et al.* [41] Their findings suggested that due to the lack of sample preparation and therefore lack of chromatographic separation, DESI-MS is vulnerable to false-positive and false-negative findings compared to conventional LC-MS and therefore, it is not reliable enough to be used in the detection of food fraud.[41]

3.2 Direct analysis in real time (DART)

DART is an ambient ionisation technique enabling non-contact analysis of solids, liquids and gaseous samples whilst producing analytical results very like that of DESI and DAPCI.[125] Helium, nitrogen or argon gases are typically used in the DART source and a corona discharge between a needle electrode and a disk electrode produces ions, electrons and metastable atoms. These are then pushed through the DART source into a grid electrode to pick up cations and anions allowing only excited state species to continue. The plasma stream is pushed through a heated electrode which can be optionally heated often helping analytes to vaporise or desorb from the substrate surface.[39] The analytes are desorbed and ionized through various ionisation mechanisms including penning ionisation (PI), proton transfer (PT), electron capture (EC) and charge exchange, all of which very much depend on the proton affinity (PA) and ionisation energy (IE) of the analyte molecules as well as the energy of the metastable atoms of the noble carrier gas.[39,45,127] This is exemplified in figure 2 which illustrates the numerous ionisation mechanisms that can occur when operating DART in both positive and negative ion mode. The types of ions that are produced also very much depends on the sample solvent, the nature and temperature of the gas.[39] Often helium is the preferred carrier gas as it produces higher energy metastable atoms than argon or nitrogen which are capable of inducing PI of atmospheric nitrogen, oxygen and water as well as most analytical solvents. Like DESI, the gas spray can be directed at an angle to the surface and then rebound at another angle towards the MS inlet or it can be aimed directly towards the MS orifice.[125] Because DART is a gas ionisation technique, this results in very few multiply charged or metal adduct ions being formed compared to that of DESI. However, a somewhat large drawback of the technique is that ion fragmentation is often observed within DART spectra when using high discharge gas temperatures which complicates raw spectra.

3.2.1 The analysis of food using direct analysis in real time mass spectrometry (DART-MS)

Compared to DESI-MS, there is substantially more literature suggesting that DART-MS is capable of analysing food samples. However, much of the published work is not centred on investigating the adulteration of food but rather on how well DART-MS adapts to different

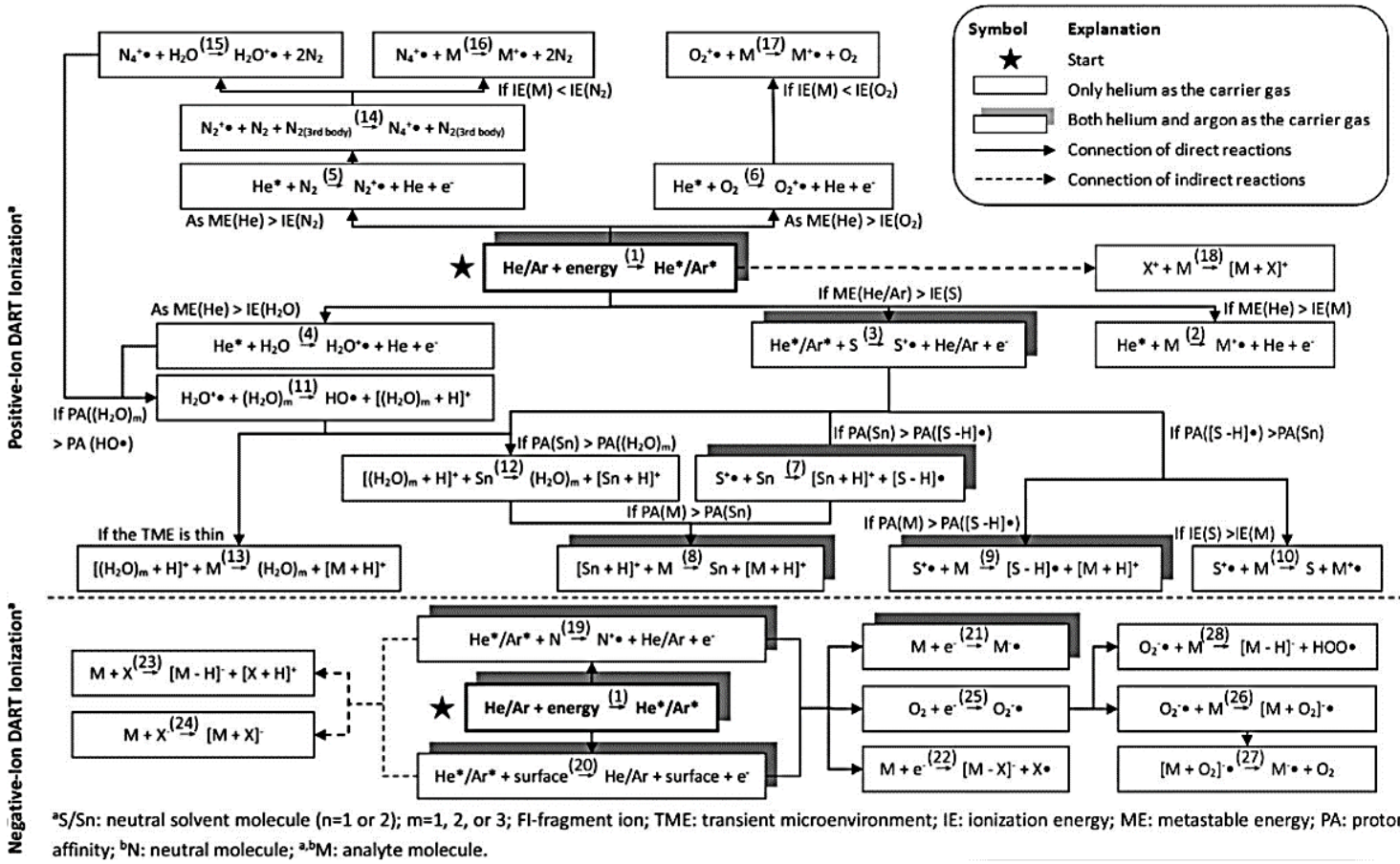


Figure 2. Ionisation mechanisms of direct analysis in real time (DART) operated in both positive and negative ionisation mode illustrating that the energy of the metastable atoms of the noble carrier gas and the proton affinity and ionisation energy of the analyte molecules and atmospheric gases have a major role in which ionisation pathway is taken.[127]

situations. An example of this type of study was undertaken by *Rahman et al.* who attempted to use DART-MS to locate the bioactive components of curcumin present in turmeric rhizomes.[128] The researchers could apply DART-MS to locate the curcumin present in the pitch of the turmeric rhizomes. These can now be extracted and added to curries and other dishes to retain the beneficial effects whilst not making the food unpalatable due to the colour or odour.

Whereas *Nielen et al*. stated that DESI-MS is inadequate at detecting food fraud, some of the literature suggests that DART-MS is much more efficient at it in such applications. Most of the work that has been carried out using DART-MS to detect the adulteration of food appears to have been led by the research group of Jana Hajslova, Tomas Cajka and Lukas Vaclavik, who have attempted to detect adulteration in many different food and drink items with varied degrees of success.[114,129]

3.2.1.1 Chicken feed

Cajka et al. investigated whether DART-MS, along with multivariate data analysis, could be utilized to assess the control of chicken feed fraud. Chicken feed normally consists of wheat, corn and soya meal along with other minor components such as barley and oat.[48] Their aim was to investigate if DART-MS could differentiate between chickens that had been fed with and without chicken bone meal using metabolomics. Polar and non-polar extracts of chicken muscle were analysed in both DART (+) and DART (-). After analysis of the data *Cajka et al.* decided to only analyse polar extracts in DART (+) and non-polar extracts in DART (-) as they provided the most complex fingerprints which were subsequently used in the analysis of a large series of chicken samples. [48] Three dominating ions were detected in the polar extracts; creatine, carnosine and anserine, whilst fatty acids (FA) were detected in the non-polar extracts.

Using both principal component analysis (PCA), an unsupervised technique and orthogonal partial least squares-discriminant analysis (OPLS-DA), a supervised technique, *Cajka et al.* where able to clearly demonstrate that by using DART-MS, they could differentiate between

chickens that had been fed with chicken feed and chicken bone meal and chickens that had been fed with just chicken feed, with both polar and non-polar fingerprints able to show this.

3.2.1.2 Dairy products

Dairy products are extremely susceptible to food fraud,[130] with the Chinese milk scandal in 2008 being the highest profile case to date. The rationale for the addition of melamine was the fact that milk prices are dictated by their nitrogen content. Melamine contains 67% nitrogen by mass and therefore, when added to milk it enhances the milks nitrogen content and thus the price. DART-MS, along with other AMS techniques such as DAPCI-MS and low temperature plasma-mass spectrometry (LTP-MS),[73,78] is a technique which has been utilised to detect the presence of melamine in milk powder. However, early studies identified an issue due to spectral/isobaric interferences. *Dane et al.* investigated the ionisation mechanisms of melamine using both helium and argon as the DART gas.[76] When using helium, the research group identified the formation of protonated 5-hydroxymethylfurfural (5-HMF). Protonated 5-HMF (m/z 127.0395) is a compound which has the same nominal mass to that of melamine (m/z 127.0732) which according to the research group results in clear spectral interferences. Additionally, *Dane et al.* observed that the relative abundance of 5-HMF increased with prolonged exposure to the DART heat source. As a result, the team attempted to repeat the experiments using argon as the DART gas, in combination with acetylacetone and pyridine reagent gases. The reasoning for this combination of gases was; (1) 5-HMF and melamine were not directly ionised by argon DART gas and (2) the combination of gases selectively ionised melamine whilst reducing the spectral interferences of 5-HMF. The ionisation mechanism started with a penning ionisation of acetylacetone resulting in cation radicals which when protonated form protonated acetylacetone. The proton transferred to pyridine which was then finally transferred to melamine. An important conclusion from *Dane et al.* was that although qualitatively melamine could be detected in milk powder samples, quantitatively much more work was required.[76]

Further publications using DART-MS to detect melamine in milk powder have been produced, an example being the work undertaken by *Vaclavik et al.* who could detect the presence of melamine and cyanuric acid at levels as low as 170 µg/kg and 450 µg/kg respectively.[77] Additionally, the limits of quantitation (LOQ) were 450 µg/kg for melamine and 1200 µg/kg for cyanuric acid. Interestingly, the research group used isotopic labelling ($^{13}C_3$ –MEL and $^{13}C_3$ –CYA) to obtain accurate quantification. However, an important observation was that deuterated melamine analogues such as MEL-d_6 were found not to be suitable for quantitative analysis.[77] *Vaclavik et al.* compared the LOQ for DART-time of flight mass spectrometry (DART-TOF-MS), LC-MS/MS (tandem MS) and ELISA when analysing melamine in dried milk, condensed milk and dried cheese samples. The team stated that there was good agreement between DART-TOF-MS and LC-MS/MS for the dried milk samples, but large differences for the condensed milk and dried cheese samples. The work undertaken by the research group demonstrated that AMS has many benefits such as lack of sample preparation, quick assay running times and comparative qualitative results to that of conventional techniques. However, quantitatively conventional techniques such as LC-MS are still much better suited.

Another means of fraud is the substitution of milk from one species with milk from another species. *Hrbek et al.* undertook the task of using DART-high resolution mass spectrometry (DART-HRMS) to investigate whether it was possible to discriminate between organic cow's milk, conventional cow's milk, goat's milk and sheep's milk.[75] When this group applied PCA to the data of all the milk samples, they made a very interesting observation. The PCA plot showed that cow's milk from both organic and conventional production were very different to sheep's milk and goat's milk. However, sheep's milk and goat's milk could not be distinguished using DART-HRMS. *Hrbek et al.* believed this occurred as the differences caused by the variability in TAG profiles were apparently larger than the inter-species differences.[75] Other interesting observations were that it was not possible to distinguish between cow's milk from organic and conventional production or possible to differentiate sheep's milk and goat's milk. However, it was possible to discriminate between cow's milk

and milk from other species using DART-HRMS. This demonstrated that ambient mass spectrometry can be utilized as a tool for the detection of at least some forms of food and drink adulteration.

Additionally, *Hrbek et al.* also wanted to further test DART-HRMS and see whether it could be used to detect plant oils in milk-based foods. In order to do this, they prepared soft cheese samples with and without rapeseed, sunflower and soybean oil. The soft cheeses were made using randomly selected cows' milk. TAG compositions in milk fat, whether it be from cow, goat or sheep are much lower compared to plant oils. Therefore, when DART-HRMS was used to detect the adulteration of soft cheese with plant oils, it could clearly detect the plant oil even to levels as low as 1 % (w/w). According to *Hrbek et al.* between the mass range of m/z 840-910 is where there is the largest contrast between authentic soft cheese and soft cheese adulterated with plant oil can be observed, due to the presence of plant $[M + NH_4]^+$ TAGs adduct ions.

3.2.1.3 Olive oil

One of the most commonly adulterated food items are oils and in particular, olive oil. This is a highly-appreciated product worldwide and is the major lipid component of the Mediterranean diet.[96] Its unique taste and flavour makes it a very desirable product and therefore it has a high price, especially the virgin products. The adulteration of olive oil has been studied extensively with many analytical techniques such as NMR, LC-MS, supercritical fluid chromatography-mass spectrometry (SFC-MS) and gas chromatography-mass spectrometry (GC-MS), but the sample preparation time in all cases is lengthy.[131-133]

There are many different grades of olive oil available, but extra virgin olive oil is the most sort after and therefore, the most expensive. As a result, it is very susceptible to adulteration. *Vaclavik et al.* utilized DART-TOF-MS to detect the adulteration of extra virgin olive oil with the cheaper hazelnut oil.[95] Using linear discriminant analysis (LDA), *Vaclavik et al.* could detect down to 6% adulteration of extra virgin olive oil with hazelnut oil.[95] Characteristic

DART-MS fingerprints in the polar TAG fractions helped identify the presence of hazelnut oil, with the time required to analyse one sample being below one minute.

3.2.1.4 Spices

Spices are commodities which have received substantial amounts of media attention in the last couple of years, and as result the spice industry is taking fraud very seriously. Cinnamon is a spice which is most commonly associated with foods, cosmetics and pharmaceuticals. With regards to cuisine, there are two types of cinnamon that are commercially sold; cassia and Ceylon (commonly known as true cinnamon). *Avula et al*. undertook the challenge of using DART-quadrupole time-of-flight-mass spectrometry (DART-QTof-MS) and PCA to investigate the authentication of true cinnamon.[89] The samples they analysed were; *Cinnamomum verum* (true cinnamon); *Cinnamomum aromaticum* (cultivated in Southern China and Burma); *Cinnamomum loureirii* (cultivated in Vietnam) *and Cinnamomum burmanini* (cultivated in Indonesia and the Philippines). The research group obtained their data in positive mode and identified clear groupings which were unique for each type of cinnamon. Between m/z 130-170 were phenylpropane compounds whilst between m/z 195-240 there were sesquiterpene compounds. There were clear differences in the DART-MS spectral data of the various cinnamon species and clear separation in the PCA plots, which according to *Avula et al.* was due to the varying intensities of coumarin, cinnamaldehyde, methyl cinnamate, aminocinnamic acid and three sesquiterpenes.[89] This work demonstrated that AMS has a very important role to play in improving the traceability and authentication of food.

When the adulteration of a food or drink commodity is undertaken, the consumer's health is seldom if ever considered by the fraudster. In some cases, the adulteration of food can have serious health implications, such was the case in the Chinese milk scandal. Work undertaken by *Shen et al.* demonstrated such a case where the power of techniques such as DART-MS can be effectively utilised.

Using DART-HRMS, *Shen et al.* investigated whether they could identify the presence of anisatin in Japanese star anise rapidly. A carpel of star anise was held in position for 15-25 s and measurements were taken in both positive and negative mode. The resulting spectra showed the clear presence of anisatin in Japanese star anise with the signals being greater than 1000 times in intensity compared to that of the Chinese star anise. The main marker of anisatin in positive mode was identified at m/z 346.148 which was the $[M+NH_4]^+$ adduct and in negative mode the marker was identified at m/z 327.107 which was the $[M-H]^-$ adduct.[88] *Shen et al.* stated that even though both ionisation modes clearly identified the presence of anisatin in Japanese star anise, the spectra produced in negative mode were higher in terms of sensitivity and had less interference.

As well as identifying clear spectral differences between Chinese star anise and Japanese star anise, *Shen et al.* also investigated whether it was possible to detect the presence of Japanese star anise in herbal teas that commonly contain star anise. *Shen et al.* spiked tea samples with Japanese star anise at concentrations of 0%, 1%, 2%, 5%, 20% and 50%. By dipping a glass rod into the tea so that approximately 2 µL was analysed, the researchers could produce calibrations and establish that adulteration at levels as low as 1% (w/w) were measurable. When carrying out a small retail survey on eight herbal teas purchased in the Netherlands, no anisatin was found. However, the work undertaken by *Shen et al*. demonstrated the importance of combating food adulteration in terms of protecting the public's health.

3.2.1.5 Fruit

Novotna et al. investigated the possibility of using DART-TOF-MS to differentiate between organically and conventionally grown tomatoes and sweet bell peppers.[110] Using PCA followed by LDA, the research group successfully separated the two different growing methods for both tomatoes and peppers. *Novotna et al.* obtained 29 and 25 markers in positive and negative ion mode respectively to build the chemometric models of tomatoes. With regards to the analysis of the pepper samples, the team obtained 32 and 58 markers for positive and negative ion mode respectively. The recognition ability of the LDA models were 97.5%

for the tomato samples and 100% for the pepper samples. However, the research team suggested that the clear differences in the statistical models was perhaps influenced more by the year of production compared to the type of farming used. As a result, they suggested that the models obtained were not reliable enough and that a larger sampling platform was required for further tests. Whether the separation was a result of farming methods or production date is not known. However, either way separation for both foods using AMS was achieved demonstrating the importance it has in the analysis of food.

3.3 Atmospheric solid analysis probe (ASAP)

ASAP is a technique that was developed in 2005 by *McEwen et al.* and offers an alternative to electron ionisation (EI) and chemical ionisation (CI).[40,125] The sample, whether it be liquid or solid, is introduced into the ionisation chamber using a glass rod and can therefore, be vaporized and ionised at atmospheric pressure.[134] A stream of hot nitrogen gas (300-500^{o}C) is used to evaporate a solid sample from the tube's surface, which is ionised at atmospheric pressure by the corona discharge of an APCI source.[125] ASAP is unique because it does not require any solvent as opposed to DESI or DAPCI and it occurs in a dry atmosphere unlike DART, which takes place in an open environment which allows atmospheric water to be part of the chemical ionisation process.[135] A major advantage of using the ASAP probe is that there is no need for a vacuum lock as the sample is introduced at atmospheric pressure.[43] Key parameters that determine the quality of ASAP-MS spectra include the corona current, the sample cone voltage and the gas desolvation temperature.

3.3.1 The analysis of food using atmospheric solid analysis probe mass spectrometry (ASAP-MS)

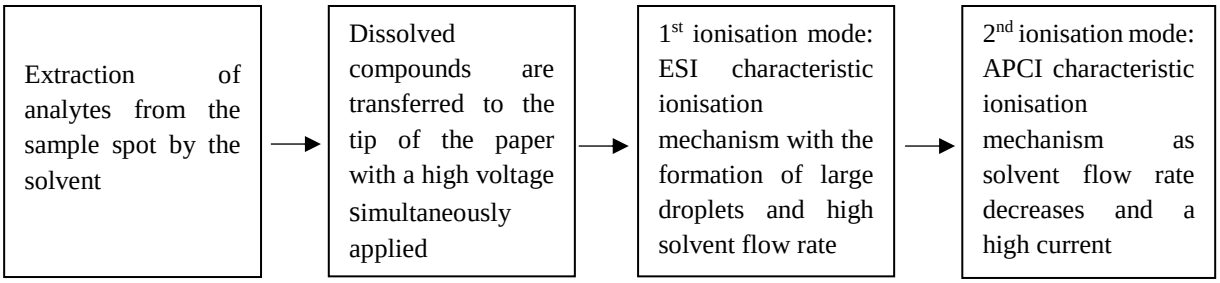
Like DESI-MS, there is a scarcity of evidence suggesting that ASAP-MS has been utilized to detect the adulteration of food, but much more widely applied to the field of pharmaceuticals and the analysis.[42] *Fussell et al.* carried out an assessment on how ASAP had been utilised in food analysis. [43] Their focus was on detecting pesticides in cereals and the detection of illegal dyes in spices.

With regards to work on spice fraud, most of the literature has been focused on the addition of Sudan dyes which are banned within the EU due to their carcinogenicity. However, there are many other illegal dyes such as malachite green and orange II available that have been found to be added to food items. F*ussell et al.* utilized ASAP-TOF-MS to detect the presence of the illegal dye auramine in saffron, which is one of the most expensive spices available on the market. The ASAP probe was stirred into the sample and desorbed. The resulting ASAP-TOF-MS spectrum produced an ion at m/z 268.1805 which corresponded to auramine $[M+H]^+$. The results were in agreement with results produced using LC-MS/MS, which verified the presence of auramine at 8 mg/kg.[43] *Fussell et al.* also stated that the ASAP probe had been used to detect the presence of bixin and norbixin in paprika, which are EU approved food additives,[136] and coumarin in cinnamon.[43] Coumarin, although found naturally in cinnamon as described previously, is also permitted to be used as a food additive. However, after investigation by the European Food Safety Authority (EFSA), a daily intake limit of 0.1 mg / kg bodyweight was set because repeated high intakes of coumarin can lead to liver failure.[137] Work was undertaken by Waters Corporation to investigate whether the ASAP probe along with a triple quadrupole (TQD) mass spectrometer could be utilized to detect melamine in a range of milk based food products.[138] 1 µl of milk, infant formula, or the supernatant from chocolate or biscuit were shaken with acetonitrile and directly loaded into onto the ASAP probe. The experiments were conducted in positive mode and a hot stream of nitrogen gas (400^{o}C) was used. According to Waters, within 2.5 minutes the ASAP probe and TQD were able to screen for the presence of melamine at levels which were relevant to legislation in a range of sample matrices. Waters Corporation set the TQD in multiple reaction monitoring (MRM) mode allowing them to acquire three transitions. Similar to the work undertaken by *Yang et al.*, Waters Corporation identified the melamine mass ion of m/z 127. The fragment ions identified using the ASAP probe were m/z 110, 68 and 60, whilst in the work undertaken using DAPCI-MS, the fragment ions identified were m/z 110, 85 and 60.[73,138] A study investigating the fragmentation of melamine was undertaken by *Ju et al.* where they identified that m/z 85 and 68 were both fragments of melamine, with m/z 85 being $[C_2N_2H_5]^+$and m/z

68 being $[C_2N_3H_2]^+$.[139] Although Waters Corporation identified fragments of melamine, there was no information regarding which, if any, food items were contaminated with melamine. Overall, ASAP-MS provides good qualitative results, but with regards to quantitative results, the technique struggles and therefore, it is insufficient at detecting the adulteration of food.

3.4 Paper-spray-mass spectrometry (PS-MS)

To some paper spray mass spectrometry (PS-MS) is widely regarded as the first ambient mass spectrometry technique by Fenn in 1998. Paper spray ionisation operates by applying a high voltage to a paper triangle wetted with a small volume of solution. When the high voltage is applied, charged droplets are emitted from the paper tip towards the MS. *Espy et al*. have investigated PS-MS extensively and found that the technique has two ionisation modes.[140] Initially, the ionisation mechanism is characteristic to that of an ESI process due to the high solvent flow rate. Multiple Taylor cone jets are created resulting in the droplet size at the beginning of the solvent flow being fairly broad (1-2 microns), with the spectra being dominated by proton-transfer reactions. As the volume of the solvent begins to deplete the size of the droplets also gradually decrease (< 1 micron). With higher currents (>0.8 μA), the spectra begin to show similarities to that of APCI suggesting a contribution from corona discharge. Another interesting observation in the work undertaken by the research group was that the drops had the same velocity regardless of the drop size. No extra gas for nebulisation is required and analytes can be analysed at low voltages (3V) when carbon nanotubes are incorporated into the paper.[141]



Workflow 1. The sample workflow and various ionisation mechanisms that occur during paper spray-mass spectrometry (PS-MS).[140]

3.4.1 The analysis of food using paper spray-mass spectrometry (PS-MS)

The literature suggests that much work has been dedicated towards the analysis of food using PS-MS. A review carried out by *Zhang et al.* demonstrated this including the identification of clenbuterol, melamine, plasticizers and dyes in meat (beef and pork), milk, chilli powder and sports drinks.[49] Additionally *Klampfl et al.* demonstrated that since 2010, food commodities such as olive oil, spices and beverages have also been investigated using PS-MS.[142] The technique has also been utilised to analyse of cola and identify pesticides in fruit and vegetable products.[108,117]

A commodity which is of huge importance to developing counties is coffee. This product is produced mostly in Asia, Africa and Central and Southern America. In 2014 Brazil was the largest producer of coffee and according to the International Coffee Organization (ICO) it was also the largest exporter in July 2015.[143,144] Most coffee is consumed in developed countries, with the EU and USA being responsible for 86% of total coffee imports.[145] *Garrett et al.* undertook the challenge of investigating whether or not is was possible to geographically discriminate between coffee beans which had originated from three different regions in Brazil using PS-MS.[118] The research group obtained arabica coffee beans, which are responsible for approximately 70% of the global coffee market, from Bahia, Rio de Janeiro and Paraná. The coffee beans were extracted in MeOH: H_2O solution (9:1) and then 5µL was spotted onto a triangular shape paper. Measurements were carried out in both positive and negative mode, but after initial review *Garret et al.* established that the spectra in negative mode were dominated by high background peaks and as a result, they only used the positive mode data. Using PCA and hierarchical cluster analysis (HCA), the research group identified three clear groupings which represented the three different geographic origins of the coffee beans. The reasoning behind the groupings was not due to identification of unique geographic markers, but instead the varying intensities of the ions.

3.5 Other ambient mass spectrometry techniques

The limited yet promising role of ambient ionisation techniques; DART, DAPCI and PS coupled with mass spectrometry have played in detecting food adulteration and food

authentication in various commodities has been outlined. However, most of the published techniques that have been utilised were around pharmaceutical sciences. In much of the literature assumptions have been made that because the technique performs well in one area of analytical science then it must be employed in different area; i.e. food safety. An example of this was shown in a paper by *Ren et al.* where they utilized high-voltage-assisted laser desorption ionisation-mass spectrometry (HALDI-MS). They established that it was capable of analysing liquid samples including proteins, pharmaceuticals and other biological fluids in both positive and negative mode.[146] They went on to state that the technique could be further developed to aid the rapid analysis of food, however, to date there is no literature concerning the use of HALDI-MS to investigate food analysis. Potentially, HALDI-MS may be like DESI-MS in that they both produce very accurate and reliable results in applications such as pharmaceuticals, but in terms of food analysis they may both suffer the same shortfalls.

3.5.1 Easy ambient sonic-spray ionisation-mass spectrometry (EASI-MS)

EASI is an ambient ionisation technique similar to DESI in the fact that it is a spray jet desorption technique. Developed in 2006 by *Eberlin et al.*, it operates by forming very minute charged droplets which are produced by nitrogen gas flowing at sonic speeds causing a statistical imbalance distribution of cations and anions.[147,148] It is thought that EASI is the simplest ambient ionisation technique as only a compressed gas (nitrogen or air) is required resulting in no need for high voltages, UV lights, laser beams, corona or glow discharges and heating.[148] This is a result of EASI being a technique which is based upon sonic-spray ionisation (SSI), a very soft ionisation technique and therefore, quite often leads to intact analyte ions being produced.[149] Positively and negatively charged droplets which are produced simultaneously strike the analyte surface. When operated in positive ion mode, protonated, sodium and potassium adducts are typically formed with deprotonated ions being formed when operated in negative ion mode.

Olive oil fraud through adulteration with cheaper oils and the detection using DART-TOF-MS has previously been discussed. Another form of fraud is based on the geographic origin

of oils that are labelled as originating from one country but instead originate from another. Therefore, it is essential that there are analytical techniques that can be utilized to detect differences between olive oils originating from different countries, hopefully through the identification of unique markers. *Riccio et al*. utilized EASI-MS with a QTof mass spectrometer to investigate whether it was possible to discriminate between thirty different olive oil samples which had originated from Portugal, Italy, Spain, Greece and Lebanon.[96]

Air dried extracts were obtained using 0.3mL oil and 1mL (MeOH: H_2O) (1:1) solution. Droplets were then placed on the sample spot and allowed to dry.[96] Using chemometrics *Riccio et al.* could clearly discriminate between the samples based on their geographic origins. Additionally, it was also possible to discriminate between the samples based on their FA ratios due to a set of four ions of m/z 255, 279, 281 and 283. Samples originating from Spain contained the greatest relative abundance of phenols, whilst the samples originating from Lebanon contained the lowest. Unfortunately, the researchers were unable to identify unique markers for all the olive oils except for the samples originating from Lebanon due to the presence of m/z 564.

A review carried out by *Porcari et al.* summarising the work that had been carried out investigating food quality and authenticity using EASI-MS identified that the technique is capable of differentiating various different types of oils.[150] Figure 3 is an image taken from the review demonstrating clear spectral differences between four different oils; olive, soybean, andiroba and acai. The main spectral differences of the oils were due to characteristic TAG fingerprints. The review also identified that food quality issues in coffee had been addressed using both EASI-MS and DESI-MS, where both techniques had successfully differentiated coffee beans treated by dry, semi-dry and wet post-harvest methods using PCA.

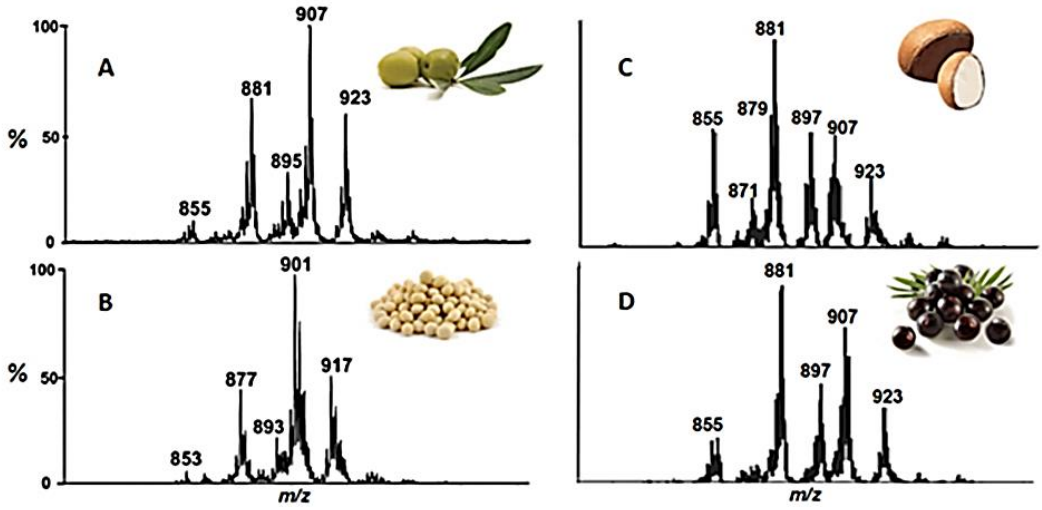


Figure 3. An image taken from a review carried out by *Porcari et al.* investigating the analysis of food quality and authenticity using EASI-MS. The image above is a compilation of EASI (+) -MS spectra of (A) olive oil; (B) soybean oil; (C) andiroba oil; and (D) acai oil.[150]

Caviar is a luxurious product which the public are willing to pay a high premium for. However, the fast-natural degradation of the product presents issues when shipping it around the globe. Therefore, conservation protocols such as salting and pasteurisation are carried out to preserve the product. However, pasteurisation is believed to reduce the culinary and economic value of caviar. Due to the high price of this luxurious product, it is susceptible to food fraud with salted caviar being substituted with pasteurised caviar. *Porcari et al.* investigated whether it was possible of differentiating the two types of caviar based upon their lipid profiles.[65] In their work three mass spectrometry techniques were utilised, with EASI-MS coupled with thermal imprinting (TI) being one of them. TI-EASI-MS is one of many variants of EASI, based on a simple lipid extraction prior to sample analysis.[150]

Caviar samples (500mg) were analysed on an envelope paper with a solution of MeOH: $CHCl_3$ (2:1, v: v) being dripped on the sample surface. Using a halogen bulb, the lipid fraction had thermally imprinted on the envelope, ready to be analysed by EASI-MS in positive ion mode. With the samples being run at both room temperature and 4°C, *Porcari et al*. stated that there were clear spectral differences between the two types of caviar at 4°C which was due to the

relative abundances of m/z 828 [PC (16:0/22:6 or 18:1/20:5) + Na]$^+$ and m/z 927 [TAG 56:7 + Na]. Pasteurised caviar had a greater abundance of m/z 927 whilst salted caviar had greater levels of m/z 828.[65] The overall conclusion stated by the research group was that TI-EASI (+)-MS was capable of comprehensive lipid profiling as both phosphatidylcholines (PC) and TAG ions could be simultaneously analysed.

Dry-cured hams are a delicacy appreciated by consumers worldwide. Produced in many regions around the world, the most famous dry-cured hams mainly originate from Mediterranean countries such as Spain, Italy, France and Portugal. Most these hams are covered by either a PDO, PGI or TSG. This can result in food fraudsters attempting to make economic gains through the fraudulent sale of dry-cured hams. The quality of these products is related to the lipids of the muscle tissues of the pigs from which they are manufactured from. Lipid characteristics are related to the rearing systems and environmental surroundings of the animals and therefore, it is believed that analytical techniques can be used to differentiate between different dry-cured hams. *Fernandes et al.* attempted this when they utilised TI-EASI-MS to differentiate between five different dry-cured hams; Prosciutto di Parma (Italian), Jamón Serrano, Jamón Ibérico de Bellota, Jamón Ibérico de Recebo, and Jamón Ibérico de Cebo (Spanish).[60] Jamón Ibérico de Bellota is believed to be the finest grade and arguably most well-known brand of Jamón Ibérico (Iberian ham) as the pigs eat only acorns. Data obtained in negative ion mode identified many FFA [M-H]$^-$ including oleic (m/z 281), linoleic (m/z 279), palmitic (m/z 255) and palmitoleic (m/z 253) acid. Differences in the ratios of the ions were evident in the Jamón Ibérico de Cebo and Jamón Ibérico de Bellota samples. Operating in positive ion mode, the research group identified that the spectra were dominated by two clusters of ion peaks, one between m/z 600-650 and another between m/z 850-950 with the latter corresponding to the presence of [TAG + Na]$^+$ adducts. The spectral profiles of the five hams were visually very similar according to the research group, however, they did state that Jamón Ibérico de Bellota revealed higher relative abundances for m/z 907 which was identified as trioleoyl-glycerol (OOO). The communication also stated that the relative abundances of ions from oleic acid and the molecules of DAG and TAG containing

oleic acid could be directly related to the pig breed and feeding characteristics.[150] Although *Fernandes et al.* could not clearly separate out the five different dry-cured hams, their work is encouraging and perhaps identifies that other analytical techniques which are suited for lipidomics based experiments could have a role to play in detecting dry-cured ham fraud.

3.5.2 Laser desorption spray post-ionisation-mass spectrometry (LDSPI-MS)

The coupling of laser desorption and ESI post-ionisation is a popular combination which has led to the creation of techniques such as ELDI, LSI, LAESI, and MALDESI. In reality, there is very little difference between these techniques, with the main point of distinction being the type of laser that is used (UV, IR, neodymium-doped yttrium aluminium garnet (Nd:YAG), etc.). The popularity of laser based techniques is down to the fact that spatial resolution is achieved and multiply charged ions are freely generated. With regards to food analysis, LDSPI-MS has not really played a key role, bar the work undertaken *Liu et al.* who investigated whether it was possible to differentiate between yogurt brands based on unique fingerprints. In their work, the research group utilised a Nd:YAG laser (wavelength of 1064nm) and irradiated the samples, which were deposited on a gold surface at 45^{o} angles. A solution of MeOH:H_2O (1:1, v:v) was introduced through a spray emitter at a flow rate of 0.2μL/min.

The research group attempted to differentiate three different brands of yogurts; Erhmann, Guangming and Yili. Working in positive ion mode, *Liu et al*. obtained spectra that showed clear visible differences between the three yogurt brands. A PCA score plot of the data emphasised those differences, with three clear groupings, each one representative of the three different yogurt brands. This work showed glimpses that laser based AMS techniques may have a key role to play in tackling food fraud. It must also be stated that MALDESI has been also been utilised in some sort of capacity to analyse food. However, this work was carried out using a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer.[151] Although the work undertaken by *Liu et al*. is encouraging, it is still some time off before it can be stated that laser based AMS techniques provide 100% accurate and reliable results with regards to detecting the adulteration and fraudulent sale of food.

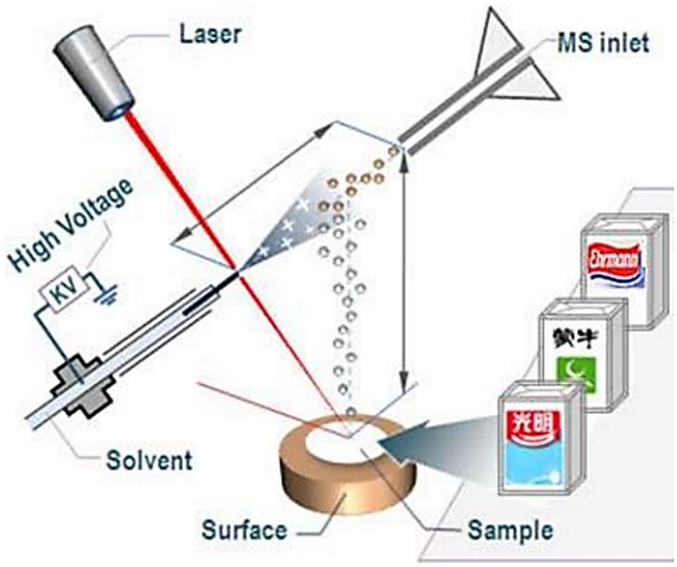


Figure 4. A schematic diagram taken from the work undertaken by *Liu et al.* demonstrating the setup and ionisation mechanism LDSPI-MS.[97]

3.5.3 Desorption atmospheric pressure chemical ionisation-mass spectrometry (DAPCI-MS)

DAPCI-MS has already been discussed in this review with positive, accurate and reliable results being obtained through the investigation of melamine and Sudan dyes in milk and tomato sauce respectively. An extremely important food group which has not been mentioned so far is fish (excluding caviar). In comparison to other commonly adulterated foods, AMS has rarely been utilised to investigate the fraudulent sale of fish, as table 4 identified. Adulteration of this product can occur in many different formats, with the common methods being based upon the species, catch method and origin of the fish. This makes it even more surprising that AMS, this exciting and new area of analytical science, is not being exploited. Other than the work carried out by *Cajka et al.* who investigated fish metabolomics and its effect on dietary supplementation using DART-MS,[63] and a study of salmon, trout and sardine using TI-EASI-MS by *Porcari et al*.,[66] the only fish fraud study using AMS has been carried out by *Wu et al.*[64] Using DAPCI-MS the research group analysed dried sea cucumber samples from three different locations within the North China Sea (Dalian, Weihai and Yantai). The team acquired the raw data in positive ion mode with the spectral information lying between m/z 50-800 and successfully differentiated between the samples using both PCA and soft independent modelling of class analogy (SIMCA).

Fish is an extremely popular product due to its nutritional benefits. *Wu et al.* findings are encouraging to some extent, but realistically AMS needs to play a much more significant role in analysing fish that; (1) are more commonly associated with our everyday lives and (2) are known to be much more prone towards food fraud, with examples being cod, tuna and salmon. Perhaps the lack of literature is an indictment of the studies that have already been undertaken using various AMS techniques and the results obtained were not satisfactory. Conventional techniques are potentially better suited for this area of food analysis, with issues such as frozen/fresh fish differentiation, fish authentication and the mislabelling of fish all being examined as stated in table 4.

4. The analysis of meat adulteration

Meat is often shown to be one of the most vulnerable commodities, particularly processed meats to food adulteration. The sale of fraudulent meat was one of the most widely discussed issues over the past three years, especially during and after the 2013 European horse meat scandal. The scale of the fraud was substantial and led to widespread decline in consumer confidence. Meat adulteration can take many forms and there are many points of vulnerability due to complex supply chains. According to *Ballin*, meat adulteration can be organised into four main areas where fraud is most likely to occur:[152]

1. Meat origin (sex, meat cuts, breed, feed intake, slaughter age, wild vs farmed meat and geographic origin).
2. Meat substitution (species, tissue).
3. Meat processing or treatment (fresh vs thawed, meat preparation)
4. Non-meat ingredient additions (water and additives).

4.1 Detection of meat adulteration

Meat adulteration can be carried out through various formats with most cases being facilitated by complex supply chains. Therefore, reliable analytical techniques are required to detect such processes. ELISA and PCR are the most commonly used for meat adulteration studies.[153,154] PCR is utilised extensively to detect the adulteration of meat because it is a DNA-based

technology. DNA is thought to be the most appropriate molecule for species detection and identification within meat and fish samples because it is highly stable and can therefore, be analysed in fresh and frozen food meat products. This is a huge advantage for PCR based experiments as the genome remains unchanged within frozen and fresh produce, whilst experiments which are undertaken using LC-MS and NMR in the main are based upon the metabolome. Metabolites within food are thought to vary drastically between freezing and thawing cycles, making the identification of unique markers which can be found in both fresh and frozen meats very difficult. This in turn can decrease the reliability of data acquired by LC-MS and NMR when detecting the adulteration of meat. Recent work undertaken by *Quinto et al.* demonstrated how effective PCR can be when they investigated the mislabelling of game meat species on the US commercial market by DNA barcoding.[51] Game meats have high costs whilst meats such as beef, pork and poultry are lower priced. Thus, there is the potential for large profits to be made through the mislabelling of game meats. *Quinto et al.* analysed fifty-four game meats products and through DNA barcoding they established that 18.5% of the products were mislabelled. According to the research group over half of the mislabelled products were a result of economic gain with the other products being a result of inadequate traceability or mishandling by the distributor or supplier.[51] The work carried out *Quinto et al.* highlighted not only that food fraud is an economically motivated concept, but also the fact that with long and complex supply chains, the chances of food fraud are drastically enhanced. Although there are benefits associated with PCR methods, at the same time there is an issue surrounding the analysis of processed meat samples. Work undertaken by *Soares et al*. has demonstrated that PCR methods provide excellent sensitivity in unprocessed foods.[155] However, with processed foods the literature suggests there is an issue regarding the sensitivity of DNA to food processing methods, partly due to the high temperatures and pH changes that are associated with processing. As the adulteration of meat is commonly associated with processed foods, this is a severe limitation.

ELISA is a plate-based assay capable of detecting proteins, peptides, antibodies and hormones. Like PCR it is a very popular technique when detecting the adulteration of meat,

as shown in the work undertaken by *Hsieh et al.*[52] The research group investigated the adulteration of raw, cooked and autoclaved beef and pork products with horse meat. They developed horse selective monoclonal antibodies (mAbs) through thermally stable horse muscle proteins and identified two mAbs that could be characterized as horse-selective. The two mAbs were H3E3 (IgG2b) and H4E7 (IgG2a). From there *Hsieh et al.* developed a suitable mAb-based ELISA and could detect the presence of horse meat down to levels lower than 1% in both raw and cooked ground beef and pork samples, and down to 0.1% in autoclaved beef and pork samples. Similar to PCR analysis, immunoassays also have their limitations, most notably the need for specific antibodies as the specificity in highly processed samples can be critical, resulting in false positive or false negative results.[14]

Proteomic studies employing mass spectrometric techniques are beginning to play a more prominent role in the detection of food fraud due to the limitations that are associated with PCR and ELISA analysis. ESI and matrix assisted laser desorption/ionisation (MALDI) are the two main ionisation sources utilised for LC-MS studies. With regards to ESI, the ability to produce multiply charged ions allows high mass proteins to be analysed and with it being a soft ionisation technique, very little fragmentation occurs allowing the analysis on intact proteins. Typically, high-resolution accurate-mass (HRAM) spectrometers such as orbitraps (FWHM ≈ 100,000), time of flight (TOF) (FWHM ≈ 50,000) and FT-ICR (FWHM ≈ 100,0000) are utilised. Compared to DNA, primary proteins are relatively stable against processing. Therefore, species specific proteins or peptide proteins can be used as markers to detect meat adulteration as demonstrated by *Watson et al.* who identified peptide markers in beef, horse, lamb and pork. The research group were able to identify the presence of one meat in another at levels of 1% adulteration.[156] Similar studies have been conducted by *Von Borgen et al.* who analysed the presence of horse and pork in beef products, in which they able to detect the presence of another meat species at levels < 1%.[157,158] LC-MS based proteomic profiling provides an alternative approach to PCR and ELISA analysis. However, a potential limitation associated with LC-MS analysis is the simultaneous elution of proteins and peptides which can lead to ion suppression.

Spectroscopic techniques have not been used to the extent of ELISA, LC-MS or PCR. However, there have been studies which demonstrate that they have the potential to be used as a fast screening technique which can detect meat adulteration. This was demonstrated by *Kuswandi et al.* and *Rohman et al.* who utilised NIRS and FTIR spectroscopy respectively. Exploiting the data to chemometric analysis enabled both research groups to investigate the adulteration of beef meatballs with pork.[50,159] Although spectroscopic studies provide fast results, their inability to identify biomarker differences between samples is a limitation that is overcome by previously mentioned techniques. Metabolomic profiling experiments are also commonly undertaken as demonstrated by *Trivedi et al.* who successfully integrated a metabolite profiling experiment using GC-MS with a lipidomic LC-MS approach.[160] Such studies are popular because a far more complete and in-depth analysis into food composition can be assessed. But, as mentioned previously, the effects of various freeze/thawing cycles can be detrimental hence why more genomic and proteomic studies occur.

Although the detection of meat adulteration has been studied extensively, various AMS techniques are beginning to play a more prominent role as identified in table 4. The studies employing the analytical platforms discussed above, excluding spectroscopic techniques, all require relatively long and in some cases complex sample preparation. Coupled with the long assay running times that are associated with those techniques, there is the possibility that AMS techniques could reduce laboratory time whilst producing results that are comparable to those obtained through the more commonly used techniques.

4.2 Liquid extraction surface analysis-mass spectrometry (LESA-MS)

Montowska et al. undertook the challenge of utilising AMS to combat meat adulteration. In their early work, they attempted to use DESI-MS and liquid extraction surface analysis mass spectrometry (LESA-MS) to detect meat adulteration.[53] LESA combines micro-liquid extraction from a solid surface with nano-electrospray mass spectrometry. This group stated that there were four key differences between the spectra of DESI-MS and the spectra of LESA-MS:[53]

- The ion intensities in the LESA-MS spectra were one to two orders higher in magnitude compared to DESI-MS.
- A more consistent signal level was observed using LESA-MS.
- LESA-MS produced more multiply charged peptides which meant that there were fewer ions above m/z 1000.
- DESI-MS produced more singly charged peptides which meant that there were ions in the m/z 1000-1600 region.

Both DESI-MS and LESA-MS were used to differentiate between five different meat species; beef chicken, pork, horse and turkey. Having undertaken data analysis through multivariate statistical software, it was stated that there was better grouping in the LESA-MS models and that the DESI-MS models were weaker, albeit the OPLS-DA plot gave satisfactory separation. It was also stated that LESA-MS gave more reproducible analysis and greater sensitivity compared with DESI-MS, which is in agreement with the findings of *Nielen et al.*[41,53] Further work was undertaken by *Montowska et al.* They could clearly discriminate between five different cooked meats (beef, chicken, pork, horse and turkey), as shown in figure 5.[54]

Having shown that different cooked meats could be distinguished, the researchers went on to attempt to identify heat stable peptide markers for each type of meat. Tryptic digests of raw and cooked meat were analysed using LESA-MS and the peptide markers were identified using targeted MS/MS. Fifteen markers were identified in the cooked meat samples and twenty-nine in the raw meat samples. According to *Montowska et al.* the reason for the reduced number of markers in the cooked samples was a result of the insolubility of protein aggregates. This was due to the conformational changes of proteins during thermal treatment, resulting in reduced digestion efficiency.[54] Having found heat stable peptide markers, *Montowska et al.* investigated what the level of detection was for LESA-MS. Samples of cooked beef were prepared and spiked with pork, chicken, turkey and horse meat at concentrations of 10%, 5%

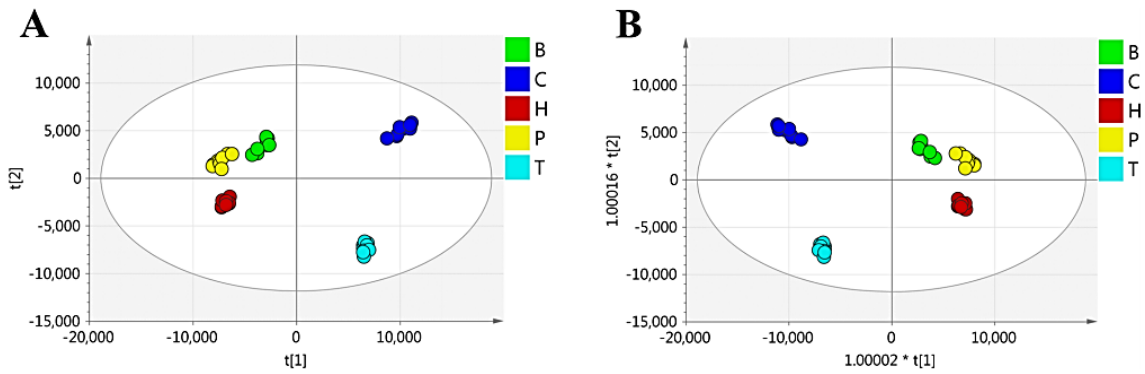


Figure 5. PCA (left) and OPLS-DA (right) plots, in the range of m/z 400-1000 taken from the work carried out by Montowska et al, demonstrating the clear separation of the five different cooked meats (beef (B), horse (H), pork (P), chicken (C) and turkey (T), using LESA-MS.[54]

and 1%. Once again, using multivariate data analysis, they could easily discriminate between the meat mixtures and demonstrated that LESA-MS successfully detected the peptide markers for horse, pork, chicken and turkey meat at 10 % adulteration. They also detected two chicken peptide markers at 5% adulteration in the beef/chicken sample.[54]

Following this work, as set of twenty-five species and protein-specific heat stable peptide markers were detected in processed samples manufactured from beef, pork, horse, chicken and turkey meat.[55] *Montowska et al.* demonstrated that several peptides which were derived from myofibrillar and sarcoplasmic proteins which were resistant to processing. A retail survey was conducted and eighteen meat products were purchased from English and Polish supermarkets. These were tested and it was found that most of the observed peptides were heat stable markers. Using the markers, they declared the meat composition of each product and identified that seven of the processed samples were a mixture of two different meat species, and one sample was found to contain offal, as shown in table 6.

Sample	Declared meat composition
Potted beef	Beef 67%, beef heart
Hunters sausage	Pork 70%, beef 20%
Kabanos sausage with cheese	Chicken 58%, pork 12%, cheese 7.5%
Pork sausage	Pork 92%, veal 6%
Cocktail sausage	Beef 60%, turkey 6%
Frankfurters poultry	Chicken and Turkey MRM 65%
Frankfurters	Veal 50%, pork 28%
Hotdogs	Pork 40%, chicken 18%

Table 6. A table identifying the meat composition of eight of the eighteen processed products analysed by *Montowska et al.* using LESA-MS.[55]

5. Quantitative analysis

This review has outlined how various AMS techniques (DART, PS, EASI, LESA and ASAP), coupled with mass spectrometry have produced qualitative results which are comparable to those obtained using conventional techniques. There is still some debate as to whether DESI-MS is suited towards detecting the adulteration or fraudulent sale of food as it has been found to be vulnerable to false-positive and false-negative results.[41] Additionally, with the lack of published literature regarding laser based AMS techniques, it is too early to suggest that reliable qualitative results can be achieved. However, food fraud or adulteration procedures cannot be reliant on just obtaining qualitative results. Some aspect of quantification, whether it be semi-quantitative, must be achieved in order to fully understand the extent of the fraud. Many food fraud incidents have shown there is a genuine risk to the public's health. The recent example of the identification of ground peanut shells and almond proteins present in ground cumin and paprika required quantification to try and understand the level of risk.[161]

It is believed that food gangs and criminals often attempt to fraudulently sell or adulterate food at levels well above 20% as any smaller amounts of substitution would not lead to substantial

economic benefits. It is well known that AMS has been perceived to provide excellent qualitative results but falls some way short in terms of acquiring accurate quantitative results. There are a few publications within this review that have demonstrated scenarios where an AMS technique has obtained quantitative results below 20% adulteration; *Vaclavik et al.* detected down to 6% adulteration of extra virgin olive oil with hazelnut oil,[95] *Shen et al.* detected the adulteration of star anise based teas at levels of 1%,[88] *Hrbek et al.* detected the adulteration of cheese with plant oils at levels of 1% and *Montowska et al.* detected chicken in beef at levels of 5%.[54,75]

Whereas most food fraud/adulteration studies have only generated semi-quantitative results, and this has been accepted, food safety is a very different issue and quantification of the risk is extremely important. Although this review has focused mainly on the adulteration, traceability and fraudulent sale of food, a small number of cases where food safety is an additional issue have been presented, providing examples of where an AMS technique has successfully obtained quantitative results. *Vaclavik et al.* successfully detected the presence of melamine and cyanuric acid in milk powder at levels as low as 170 µg/kg and 450 µg/kg respectively using DART-MS and isotopically labelled standards.[77] Using DAPCI-MS, *Yang et al.* could identify melamine in both milk powder and liquid milk at levels of 1.6 e^{-11} g/mm^2 and 1.3 e^{-12} g/mm^2 respectively and *Huang et al.* could detect melamine at levels of 6-15 µg/kg in milk powder, soy milk powder, liquid milk and synthetic urine when using LTP-MS.[73,78] *Zhang et al.* could detect melamine in milk powder and infant formula at levels of 20 ng/ml and 50 ng/g respectively, illegal Sudan dyes in chilli powder at levels between 50-100 ng/g and various contaminants in beef and pork samples between 1-5 ng/g using PS-MS.[49] The work undertaken by *Fussell et al.* using ASAP-MS to detect auramine in saffron also demonstrated some potential signs of quantification when they detected the illegal dye at levels of 8 mg/kg.[43] However, it is clear that ASAP-MS struggles in terms of quantitation, as acknowledged by *Fussell et al.* and in terms of limits of detection (LOD) it is trailing behind the studies using DART-MS, DAPCI-MS, LTP-MS and PS-MS.

At present conventional and AMS techniques are providing similar qualitative results with regards to detecting food fraud. With the fact that the AMS techniques require minimal to no sample preparation and very fast assay running times compared to that of conventional techniques, AMS has a major role to play. However, in terms of quantitation there are still big issues concerning how accurate the results are and the possibility for false negative and positive results. Another issue concerning AMS techniques is that all the studies which have been shown to provide some levels of quantification are liquid based samples, or solid samples diluted/dissolved in a liquid solution. Thus, perhaps the biggest drawback of all for AMS is that it is not possible to achieve quantification of solid samples. In order to ensure that fit for purpose, reliable and accurate quantification of liquid samples and perhaps solid samples can be achieved by AMS, substantial thought and effort will have to be placed on appropriate quality control procedures as described previously by *Hajslova et al.* (spiked samples, certified reference materials and comparisons with chromatography based methods).[129] Although their recommendations are specifically described for DART-MS experiments, their suggestions can be extrapolated for any AMS based technique. Until enough studies have been carried out operating in accordance with these quality control procedures, it is impossible to know whether AMS can produce both and qualitative and quantitative results.

6. Analysis of other food related issues using AMS

Food safety, authenticity and adulteration are three terms with slightly different interpretations. Examples of adulteration, authenticity and traceability have been demonstrated thus far and although this thesis is based primarily on these issues, there are other aspects of food analysis that has been investigated using AMS. Food safety can be applied to all food analysis, whether it be the presence of mycotoxins, pesticides, fungicides, melamine, salmonella etc. AMS has played a pivotal role in analysing many of these issues. DART-MS has been successfully implemented in analysing mycotoxins in cereals and beer.[103,162] The scrutiny of pesticides in fruit and vegetables has been studied using PS-MS,[108] low temperature plasma-mass spectrometry (LTP-MS)[107] and liquid

chromatography/dielectric barrier discharge ionisation-mass spectrometry (LC/DBDI-MS).[109] The latter two techniques have fairly similar ionisation mechanisms to that of DART. The identification of fungicides in wine has been investigated using LTP-MS.[116] As mentioned earlier, the presence of pesticides in cereals has been looked at by *Fussell et al.* using ASAP-MS[43] and the analysis of lipids in butter products, the identification of triglycerides (TG) in edible oils and margarine and butter cholesterol levels have been investigated using both DESI-MS and DAPPI-MS.[84,85]

7. Conclusions

The sale of fraudulent and adulterated food is being reported widely on a global basis and much more frequently than previously. It is clear the driver for such fraud are the large profits that can be achieved. Economically motivated adulteration of food is a common practice that has been carried out since the trading of food commodities began. However, recent scandals such as the adulteration of oregano with olive and myrtle leaves,[94] and more high-profile scandals including the European horse meat scandal in 2013 have further highlighted the extent at which it is occurring. The rapid growth of ambient ionisation techniques coupled with mass spectrometry is exciting with over thirty different techniques now available. Perhaps not all can detect the adulteration of food, however, to date several ambient ionisation techniques such as DART, DAPCI, EASI, LESA and PS coupled with mass spectrometry have been proven to enhance and aid the way in which the detection of food adulteration is undertaken. Compared to conventional techniques such as LC-MS, NMR, ELISA, PCR and various spectroscopic techniques which were commonly used to investigate the adulteration of food, the authenticity and traceability of food and general food safety, these ambient mass spectrometry techniques require no sample preparation and minimal sampling time thus producing fast and accurate results which most importantly are comparable with results obtained from conventional techniques. It is clear there is rapid growth in the use of ambient mass spectrometry applied to food adulteration issues. It appears to be a technique that lends

itself to the needs of regulators and industry and may become one of the most important analytical tools in detecting food fraud globally.

8. Aims of thesis

The overall aim of this thesis is to assess whether mass spectrometric platforms, predominately rapid evaporative ionisation mass spectrometry (REIMS), in conjunction with chemometrics can be used to detect food fraud and identify what applications the techniques prosper in and what limitations are associated with them.

8.1 Objectives of each thesis chapter

The objective of chapter 2 is to determine the use of a two-tier system employing liquid chromatography-high resolution mass spectrometry (LC-HRMS) and Fourier-transform infrared (FTIR) spectroscopy to assess the potential adulteration of oregano, a culinary herb which is prone to the addition of so called bulking agents. The adulterants under investigation within this study are cistus leaves, hazelnut leaves, myrtle leaves, olive leaves and sumac leaves. FTIR will act as a fast screening technique whilst the combination of LC-HRMS and chemometric analysis will potentially enable the identification of unique biomarkers for each adulterant.

The objective of chapter 3 is to utilise REIMS to investigate whether fish speciation, one of the ‘seven sins’ associated with fish fraud can be detected near-instantaneously without the need for any form of sample preparation. The five white fish species under investigation are cod, coley, haddock, pollock and whiting. Chemometric models generated from the REIMS raw spectrometric data will be used to assess whether the species of a sample can be assigned near-instantaneously and how the time taken to assign a species classification compares to DNA techniques that are commonly associated with such studies. Additionally, the catch method (another of the ‘seven sins’ of fish) of haddock samples (line v trawl caught) will investigated to ascertain whether the REIMS technology can differentiate between the two catch methods and if so what ions are responsible.

The objective of chapter 4 is to identify what the quantitative abilities of the REIMS technology are when analysing beef burgers that are adulterated with three different meat species; goat, lamb and pork. Making burgers at adulteration levels ranging from 0.1-50%, will enable me to assess what LOD are capable and how they compare to techniques that have been used for similar such studies.

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2. A comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach

Black C, Haughey SA, Chevallier OP, Galvin-King P, Elliott CT. A comprehensive strategy to detect the fraudulent adulteration of herbs: The oregano approach. *Food Chem*. 2016; 210:551-557.

2.1 Abstract

Fraud in the global food supply chain is becoming increasingly common due to the huge profits associated with this type of criminal activity. Food commodities and ingredients that are expensive and are part of complex supply chains are particularly vulnerable. Both herbs and spices fit these criteria perfectly and yet strategies to detect fraudulent adulteration are still far from robust. An FT-IR screening method coupled to data analysis using chemometrics and a second method using LC-HRMS were developed, with the latter detecting commonly used adulterants by biomarker identification. The two-tier testing strategy was applied to 78 samples obtained from a variety of retail and on-line sources. There was 100% agreement between the two tests that over 24% of all samples tested had some form of adulterants present. The innovative strategy devised could potentially be used for testing the global supply chains for fraud in many different forms of herbs.

Keywords – Oregano; authenticity; adulteration; Fourier transform infrared; high resolution mass spectrometry; biomarkers.

2.2 Introduction

Globally, herbs and spices play a significant part in the diets of many as they are important ingredients in a multitude of foods, beverages, medicines and cosmetics. With consumers having greater access and a desire to use these products, the demand has increased vastly over the last thirty years making it a multibillion dollar industry.[1] Marieschi et al. stated that the global herb and spice trade was worth $2.97 billion with the EU market amounting to 520 thousand tonnes and a value of €1.8 billion.[2] Sales in 2014 at all United Kingdom (UK) supermarkets were £173 million for dried herbs and spices, and £107 million for fresh herbs (spices not included).[3] Economically motivated adulteration (EMA) of food is a common concept, which has occurred within the food industry since trading began.[4] As is the case with any food commodity, there is a greater possibility of food adulteration when the demand and prices increase and when complex supply chains are involved. Herbs and spices fulfil all these criteria.

Herbs and spices are two very different food items with spices tending to be bright vibrant colours emanating often from warm climates in a diverse form e.g. cumin and turmeric and herbs are usually green leaved and derived from plants in cooler environments e.g. parsley and thyme. Supply and demand is a fundamental economic principle which determines the price of all commodities. However, as well as this, the price of spices is also dictated by the intensity of their colours and therefore, common adulteration of spices had been the addition of illegal dyes such as Sudan dyes, which are group 3 genotoxic carcinogens.[5,6] However, since this issue was highlighted in 2003, the addition of Sudan dyes as a food additive has been banned worldwide. Herbs are not traded on colour and so there is no economic advantage gained from adding dyes. Instead, the price of herbs is dependent on how compact the product is and therefore bulking agents have been used as most commercially sold herbs tend to be either chopped or ground. Consequently, it is relatively easy to add other cheaper ground bulking agents without the supply chain and indeed the consumer noticing.

Oregano is a culinary herb most commonly associated with pizzas and other Mediterranean dishes. The main producers of oregano reside in the United States of America, Mexico, Greece and Turkey. Compared to most herbs, oregano has a complicated history as the true identity of it is very difficult to define. This is partly due to the large heterogeneity of the *Origanum* genus, but also due to the grouping of different botanical genera*; Origanum (lamiaceae)* from the Mediterranean and *Lippia (verbenaceae)* from Mexico.[2] Due to the confusion, this led to a clear market distinction between Mediterranean and Mexican oregano, with both having different cleanliness specifications such as the addition of sumac leaves.[7] Mexican oregano has a much stronger and robust flavour compared to Mediterranean oregano, which could be due to the varying percentages of essential oils within the leaves. The essential oil percentage in Mexican oregano leaves is around 3-4%, whilst the percentage within Mediterranean oregano leaves is around 2-2.5%.

Even with the clear commercial distinction between Mexican and Mediterranean oregano, there are still several different definitions regarding Mediterranean oregano. The European Pharmacopoeia (PhEur) and the European Spice Association only allow *Origanum vulgare L. ssp. hirtum* and *Origanum onites L.*, to be marketed as true oregano with impurities of extraneous materials of up to 2% being considered tolerable.[8-12] However, ISO/FDIS 7925 allow leaves of all *Origanum* genus, species and subspecies except *Origanum majorana L*, to be marketed as oregano.[13] Impurities of up to 1% are considered tolerable, which is in line with the value accepted by American Spice Trade Association.[3,7]

There has been a range of different detection methods developed and used in the determination of the authenticity of food. In a review by Reid et al. many methods of detections were scrutinised including spectroscopy (ultraviolet-visible (UV), near infrared (NIR), mid infrared (MIR), Raman), isotopic analysis, chromatography, electric nose, polymerase chain reaction, enzyme-linked immunosorbent assay and thermal analysis – all of which are techniques that have been applied to food authentication since 2001.[14] Food fingerprinting is of particular interest as a method of detection. Ellis et al. published a review of some of the fingerprinting technologies, with particular interest paid to NIR, MIR and Raman spectroscopic techniques.[15]

Few analytical methods are available to screen a large number of dried plant materials quickly and the detection of oregano adulteration has been limited to a few techniques. Marieschi et al. applied a random amplified polymorphic DNA (RAPD) method which has led to the development of sequence-characterized amplified region makers (SCARs) for a number of potential adulterants; *Rhus coriaria L., Cistus incanus L., Olea europaea L., Rubus coriaria L.*, which lack a clearly detectable essential oil profile and *Satureja montana L., Origanum majorana L.*, which belong to the lamiaceae family.[2,11,12,16] Work has also been carried out by Bononi et al. who have utilized liquid-chromatography mass spectrometry (LC-MS) and gas-chromatography mass spectrometry (GC-MS) to identify the presence of olive leaves in ground oregano, using oleuropein as a marker.[17-18]

The aim of this study was to develop and fully validate a two-tier approach utilising Fourier-Transform Infrared spectroscopy (FTIR) and Liquid Chromatography High Resolution Mass spectrometry (LC-HRMS) to screen for and confirm oregano adulteration. When these two techniques are combined with multivariate data analysis software they have the ability to process a large number of samples.[19-20] By applying FTIR and LC-HRMS the ability to produce the world's first comprehensive testing system of oregano adulteration was trialled. A survey of commercially available oregano samples in the UK were then undertaken to determine the current level of abuse.

2.3 Materials and methods

2.3.1 Sample collection and preparation

Samples of oregano with full provenance and traceability and a number of previously identified adulterants (olive leaves, myrtle leaves, sumac leaves, cistus leaves, hazelnut leaves), were sourced from different parts of the world. Commercially available oregano samples were purchased at various retailers including convenience shops, supermarkets and market places in the UK and Ireland. In addition, samples were also purchased from online retailers a small number that were obtained from EU and non-EU countries. The samples were milled to a homogeneous powder on a PM-100 Retsch Planetary Ball Mill (Haan,

Germany) by weighing approximately 5g into grinding jars and milling at 500 rpm for 5 minutes.

For LC-HRMS analysis, milled homogenate herb sample (0.05g) was extracted in 2 mL of methanol/water solution (1:1, v/v), mixed for 10 minutes, sonicated for 15 minutes at maximum frequency in a water bath at room temperature, centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant collected (1 mL). The supernatant was dried under vacuum and reconstituted in 1.5 mL of ultra-pure water. Subsequently, the extract was filtered through a 0.22 µm Costar Spin-X Centrifuge Tube Filter (10,000 g at 4°C for 10 minutes). Filtered extracts were immediately transferred into Waters maximum recovery vials for UPLC-QTof-MS analysis.

2.3.2 Spectral Data Acquisition using Fourier-Transform Infrared (FTIR)

For FTIR, the milled samples were placed in the ATR sample area of a Thermo Nicolet iS5 spectrometer (Thermo Fisher Scientific, Dublin, Ireland) equipped with ATR iD5 diamond crystal and ZnSe lens and DTGS KBr detector. The slip-clutch pressure tower is applied to the sample and tightened until the correct pressure was utilised which gives more reproducible results. Each spectrum was acquired in the 550-4000 cm^{-1} range. The acquisition parameters were: number of sample scans: 32; collection length: 47 s; resolution: 4.000; levels of zero filling: 0, number of scan points: 12415; laser frequency: 11742.96 cm^{-1}; apodization: N-B Strong; phase correction: mertz; number of background scans: 32; background gain: 4.0. The acquisition was repeated 3 times. Spectral data for each sample was averaged before further data processing.

2.3.3 Chromatographic and mass spectrometry conditions

Analyses were carried out on a Waters Acquity UPLC I-Class system (Milford, MA, USA) coupled to a Waters Xevo G2-S QTof mass spectrometer (Manchester, UK) with an electrospray ionisation source operating in positive or negative mode with lock-spray interface for real time accurate mass correction. Instrument settings were as follow: source temperature was set at 120ºC, cone gas flow at 50 $L.h^{-1}$, desolvation temperature at 450ºC, and desolvation

gas flow at 850 $L.h^{-1}$. The capillary voltage was set at 1.0 kV in positive mode and 1.5 kV in negative mode, respectively. Source offset was 60 (arbitrary unit). Mass spectra data were acquired in continuum mode using MS^E function (low energy: 4 eV; high energy: ramp from 15 to 30 eV) over the range m/z 50-1200 with a scan time of 0.08 s. A lock-mass solution of Leucine Enkephalin (1 ng μL^{-1}) in methanol/water containing 0.1% formic acid (1:1, v/v) was continuously infused into the MS via the lock-spray at a flow rate of 5 µL min^{-1}.

The chromatographic separation was conducted on an Acquity HSS T3 column (100 mm x 2.1 mm, 1.8 µm). The column oven temperature was set at 45ºC, injection volume at 5 µL and flow rate at 0.4 mL min^{-1}. Mobile phase consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. The gradient was set as follows: 1.50 min of 99% (A) followed by a linear increase from 1 to 99% (B) over 15 min, isocratic cleaning step at 99% (B) for 2 min, then returned to initial conditions 99% (A) over 0.25 min and column equilibration step at 99% (A) for 1.25 min. Each sample was injected three times in order to assure reproducibility. Prior to all analyses 10 pooled conditioning samples were injected. For quality control pooled samples were injected at intervals of every 10 samples throughout the entire experiment to determine the chromatographic reproducibility of retention times and peak intensities.[21-22]

2.3.4 Data processing and data analysis

Principal Components Analysis (PCA), an unsupervised technique, and Orthogonal Partial Least Squares Discriminate Analysis (OPLS-DA), a supervised technique, were used for building the qualitative models in this investigation as previously described.[23]

The generation of calibration models was carried out using similar methodology as previously published.[24] The data pre-processing included standard normal variate technique (SNV), which compensates for differences in pathlengths due to scattering effects, 2nd order derivative and Pareto scaling using the SIMCA 14 chemometric software. Spectral data was analysed between the wavenumber ranges 550-1800cm^{-1} and 2800-3999cm^{-1}. The data generated include R2 which is an estimate of the fit of the model and Q2 which is an estimate of the

predictive ability of the model and it is calculated by cross-validation. The latter is calculated by removing each 1/7th of the data in succession and building a new model on the remaining data with the omitted data predicted using this method of cross validation. Predicted Residual Sum of Squares (PRESS) is calculated by comparison with the original data with the best predictability of the model indicated by a low value. The SIMCA 14 chemometric software automatically converts PRESS into Q2 to resemble the scale of the R2 with good predictions having high Q2.

Raw data generated by the mass spectrometer were imported to Progenesis QI 2.0 software (Waters, Newcastle, UK). After data conversion to the appropriate format using a filter set at 2, data were aligned to the best pool sample selected and peak picking from 0.5 to 17.5 minutes was carried out with sensitivity set at automatic and chromatographic peak width to 0.08. The analysed spectral data were then exported to SIMCA 14 for multivariate analysis. As a quality control measure all the spectral data were Centre Scaled and analysed using PCA. All pooled samples (QC) were found to be tightly clustered within the centre of each representative scores plot which indicates good reproducibility of the data. Following this, all data were mean centred, Pareto scaled and grouped into Adulterant and Oregano prior to OPLS-DA. R^2 (cumulative), Q^2 (cumulative) and Root Mean Squared Error of cross validation (RMSECV) were used to determine the validity of the model. R^2 (cum) indicates the variation described by all components in the model and Q^2 is a measure of how accurately the model can predict class membership.

2.4 Results and discussion

2.4.1 FT-IR spectroscopic analysis

FTIR is a technique that is based on the absorbance of light at particular wavelengths and has been a popular methodology in detecting food adulteration. In a review by Rodriguez-Saona & Allendorf, examples of using FTIR in conjunction with multivariate analysis has been applied for authentication of herbal products, fruit juices, agricultural products, edible oils, dairy, and numerous other food products.[25] In this study, the powdered oregano and adulterant

samples (olive leaves, myrtle leaves, hazelnut leaves, cistus leaves, sumac leaves) were analysed on the FT-IR spectrometer. Figure 2.1 shows the FT-IR spectra of pure oregano, olive leaves and myrtle leaves. Although there was some overlap observed between the peaks in the spectrum of the oregano and the peaks in the spectra of the adulterants, visually there are observable differences in the fingerprint region from 900-1800 cm^{-1}. FTIR peaks are attributed for stretching and bending vibrations that characterize the functional groups. The regions of interest included: (i) 1100-1400 cm^{-1}, generally the most prominent peak, is due to the vibration peak of C–O in alcohol hydroxyl group (ii) 1400-1500 cm^{-1} corresponding to C–O and C–C stretching vibrations specific to phenyl groups; (ii) 1500-1600 cm^{-1} corresponds to aromatic vibrations and N-H bending and (iv) 1600-1740 cm^{-1} corresponding to bending N-H, C=O stretching (aldehydes, ketones, esters, free fatty acids and glycerides). To further observe the influence of adulterants on the spectrum of oregano, the latter was adulterated in 10% additions (0-100%) of olive leaves and the spectra recorded. Figure 2.2 shows the resulting spectra indicating the monotonic increase intensity exemplified by the peak shown in the inset. Due to these differences identified in the spectral data it was possible to apply chemometric modelling for discriminant analysis. The chemometric software (SIMCA 14) was used to generate a qualitative model using PCA (unsupervised) and supervised OPLS-DA (supervised) algorithms with Pareto scaling to determine if it was possible to differentiate pure oregano from its adulterants. The data was pre-processed using SNV, 2^{nd} order derivative algorithm with Savitzky-Golay smoothing (11 point window and 2nd order polynomial). For the unsupervised PCA model, the first four principal components describe most of the variation (84.5%) as follows: PC1 43.3%; PC2 19.4; PC3 14.5%; PC4 7.3%. Separation was achieved mainly along PC1 and PC2 with the positive scores related to oregano samples and negative scores associated with the adulterants. The measure of fit (R2) of this PCA model was 94% and the measure of predictive ability (Q2), based on cross validation was 86%. For the supervised chemometric model, OPLS-DA was used with the same pre-processing parameters used for the PCA plot. The OPLS-DA model generated one predictive components and one orthogonal components which explained 34.8% and 23.6% of the differences

respectively. The measure of prediction (Q2), based on cross-validation, was 95.9% and RMSECV=9.7%, indicating very good predictability of the data. Figure 2.3A shows PCA and OPLS-DA scores plots, and indicated that this method could be used for discriminant analysis and this approach to rapidly screen for adulteration of oregano is sufficiently robust and thus fit for purpose.

2.4.2 High resolution mass spectrometric analysis

LC-HRMS is a technique that has been used extensively for metabolic profiling in both the food and plant industries.[26-28] Profiling analysis of oregano and adulterant samples (olive leaves, myrtle leaves, hazelnut leaves, sumac leaves, and cistus leaves) was carried out using an untargeted analysis approach on a Waters UPLC coupled to a G2-S QTof mass spectrometer. One of the advantage of untargeted analysis by high resolution mass spectrometry in combination with chemometrics is the possibility to build models on an untargeted basis, with subsequent exploration of the data to discover the characteristic markers that contribute most significantly to the classification. Furthermore, recent improvements in instrumentation and processing software allow a faster, more reproducible and more comprehensive data analysis. In our case, up to 4500 ions in each ionisation mode were reliably detected along the chromatographic gradient (Figure 2.4). The extracted data were then exported to chemometric software to be subjected to similar data treatment as was the spectroscopic data presented above. The PCA score plot generated (Figure 2.3B) showed clear discrimination between the pure oregano and the adulterants, with the oregano samples clustered together on one side of the plot and the adulterant samples scattered on the other side of the plot. Additionally, there was clear separation between the two oregano species which can be marketed as Mediterranean oregano; *origanum vulgare* and *origanum onites*, with one sample which contained both species situated in-between the two groups. OPLS-DA was then performed and a model was generated with one latent component and three orthogonal components with resulting R2 = 99.7%, Q2 = 94.4% and RMSECV of 10.3% for the positive mode ionisation data and another one with one latent component and three orthogonal

components with resulting R2 = 99.3%, Q2 = 94.9% and RMSECV of 9.4% for negative mode. Several other individual models were subsequently produce by comparing Oregano to each individual adulterant (olive, myrtle, hazelnut, cistus, sumac) with their respective S-plots to enable the potential identification of markers to each adulterant (Table 2.1). This methodology allowed the identification of 16 unique markers in positive mode and 12 in negative mode, with all adulterant samples having at least 4 unique markers. This data will be used for the future development of a targeted method using MS/MS analysis.

2.4.3 Survey of commercial samples

To test the models developed for both analytical methodologies, a survey of oregano was carried out which included samples from retailers, service sector, internet sources and a few bought from commercial outlets in countries outside UK/Ireland. The spectral data generated for these samples from the FTIR and LC-HRMS were predicted as unknowns using the relevant OPLS-DA model produced earlier.

Similar predictions were obtained from the models generated using both analytical techniques. Furthermore, identified markers from LC-HRMS data were found in all commercially adulterated samples and reinforce the potential of these markers for potential targeted applications. Table 2.2 shows the results of the survey of commercially available oregano based on the spectroscopic and spectrometric data. The samples have been broken down into those procured from the retail and service sectors in UK/Ireland and those purchased on the internet or at commercial outlets outside of UK/Ireland. The results show that approximately 24% of the oregano samples tested were adulterated and the scale of adulteration ranged from 30% to over 70%, indeed two samples had virtually no oregano present. The scale and level of the adulteration uncovered was not expected. In addition the similar figures for ‘store’ bought and ‘internet’ bought were surprising as one would have thought the retail trade would have better systems of control in place. The most common adulterants found in the samples were olive leaves and myrtle leaves. The results of the survey were passed to the regulator to alert the affected companies to the economically motivated fraud ongoing in the sector. Based

on the results of this survey the samples which were indicated to be oregano by both methods can be included in the calibrations models to increase the robustness of the test.

2.5 Conclusions

The detection of fraud in foods and food ingredients has become an even more important topic since the horsemeat scandal of 2013. Many consumers lost faith in the food they were purchasing and the food industry recognised that more robust measures in terms of auditing and testing had to be put in place. Often fraud is perpetrated in high value food commodities and those which come via complex supply chains. Probably herbs and spices fit these characteristics more than any other food ingredients and are thus highly vulnerable. Testing methods for the food industry must be easy to use, rapid and low costs. Our two-tier system of testing provides not only a cost-effective means of testing but one also that will survive rigours of a legal process. The survey data presented is disturbing in the level of adulteration found. It is clear that a serious level of fraud is being perpetrated and that bona fide businesses and consumers are being financially harmed. It is likely similar (if not worse) levels of fraud are occurring in many global regions. We believe the system we have developed and validated for oregano should be expanded to cover all herbs sold in the market. Only then will there be a sufficient deterrent in place to stop fraudulent activity in these widely consumed food ingredients.

Acknowledgements

The research undertaken was funded by a BBSRC CASE studentship BBSRC BB/M0503162/1, Invest NI Proof of Concept Project PoC452 and EU FP7 FOODINTEGRITY project (agreement no: 613688).

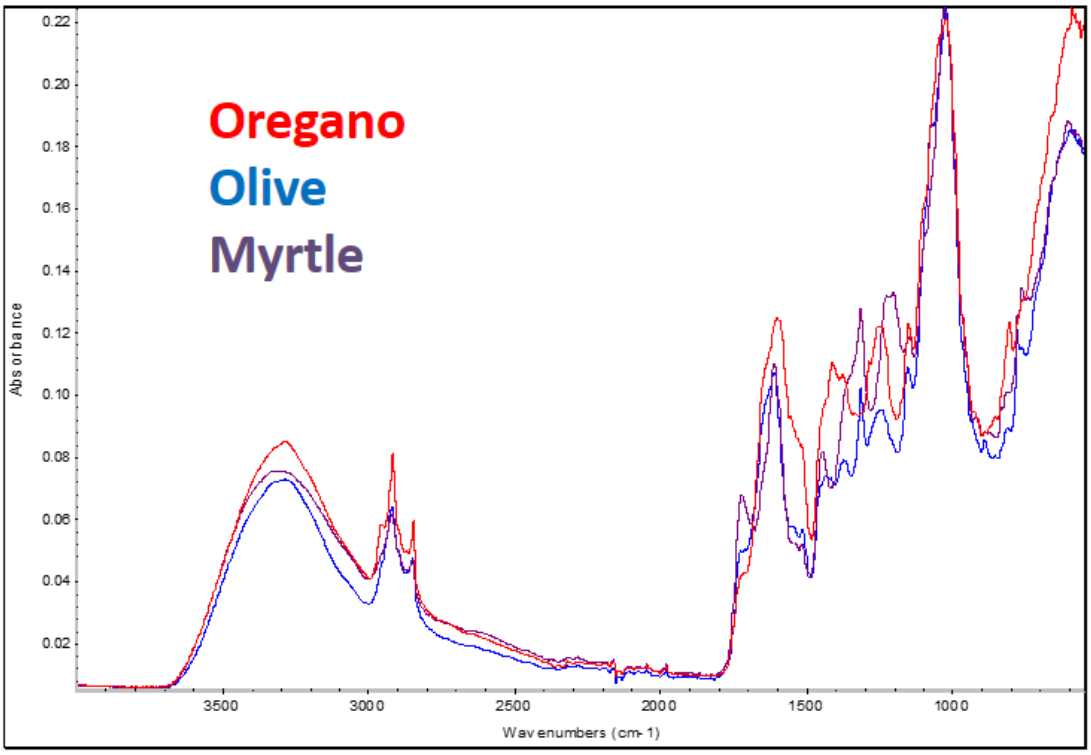

Figure 2.1. FT-IR spectra of oregano and the adulterants olive leaves and myrtle leaves. Clear spectral differences can be seen between the oregano and adulterant samples in the fingerprint region (900-1800 cm^{-1}). The regions of interest include: (i) 1100-1400 cm^{-1}, due to the vibration peak of C–O in alcohol hydroxyl group (ii) 1400-1500 cm^{-1} corresponding to C–O and C–C stretching vibrations specific to phenyl groups; (ii) 1500-1600 cm^{-1} corresponds to aromatic vibrations and N-H bending and (iv) 1600-1740 cm^{-1} corresponding to bending N-H, C=O stretching (aldehydes, ketones, esters, free fatty acids and glycerides).

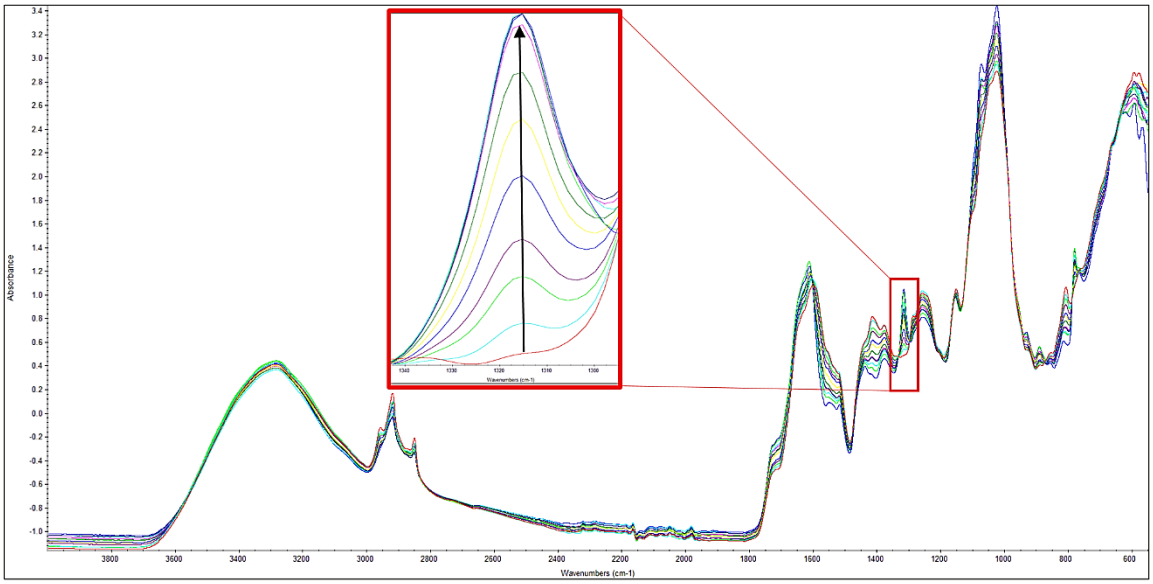

Figure 2.2. FT-IR spectra of Oregano adulterated with olive leaves in 10% additions (0-100%) showing a monotonic increase in intensity exemplified by the inset with the arrow indicating the increase in olive leaf adulteration.

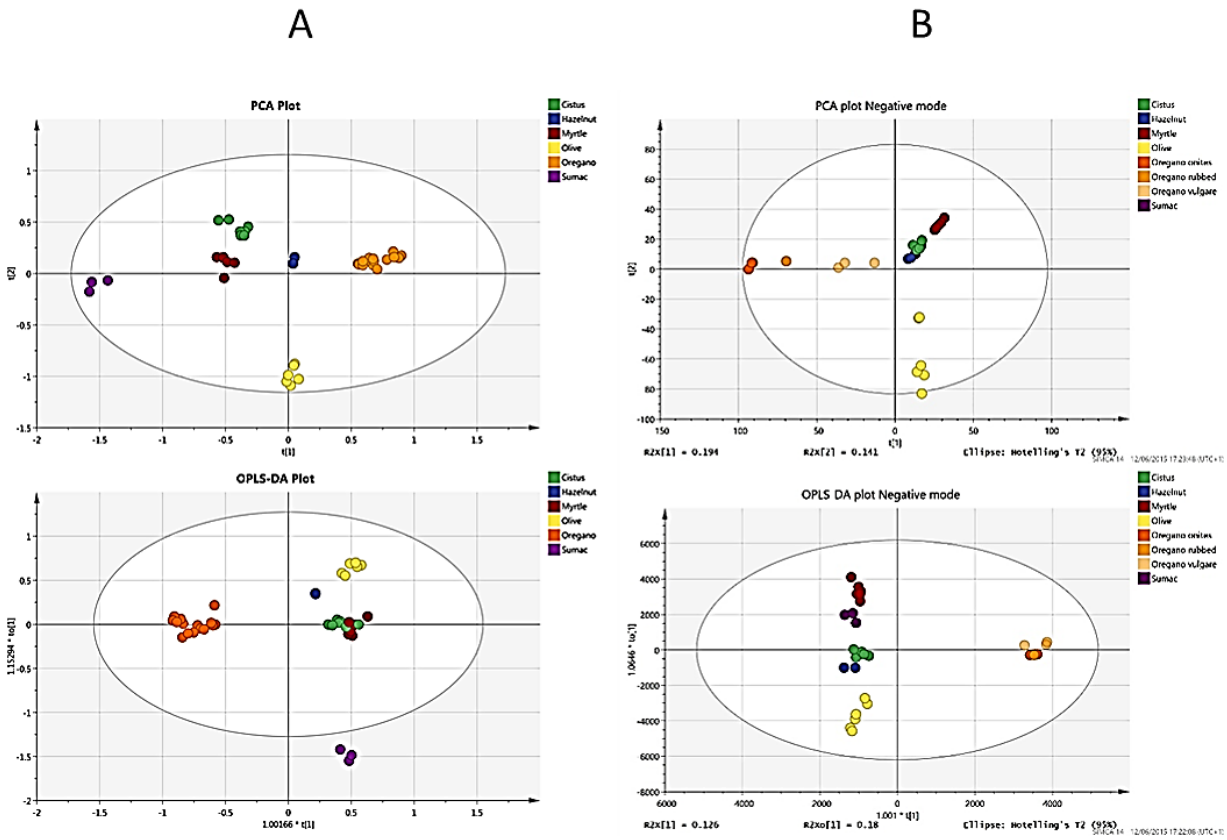

Figure 2.3. (A) Unsupervised PCA and Supervised OPLS-DA scores plots from FTIR spectral data; (B) Unsupervised PCA and Supervised OPLS-DA scores plots from LC-HRMS data in positive ionisation mode. Models generated based upon data acquired from both analytical platforms exhibit clear differences between the oregano and adulterant samples, with separation of the two commercially marketable oregano species (oregano onites and vulgare) being evident within the LC-HRMS PCA and OPLS-DA score plots.

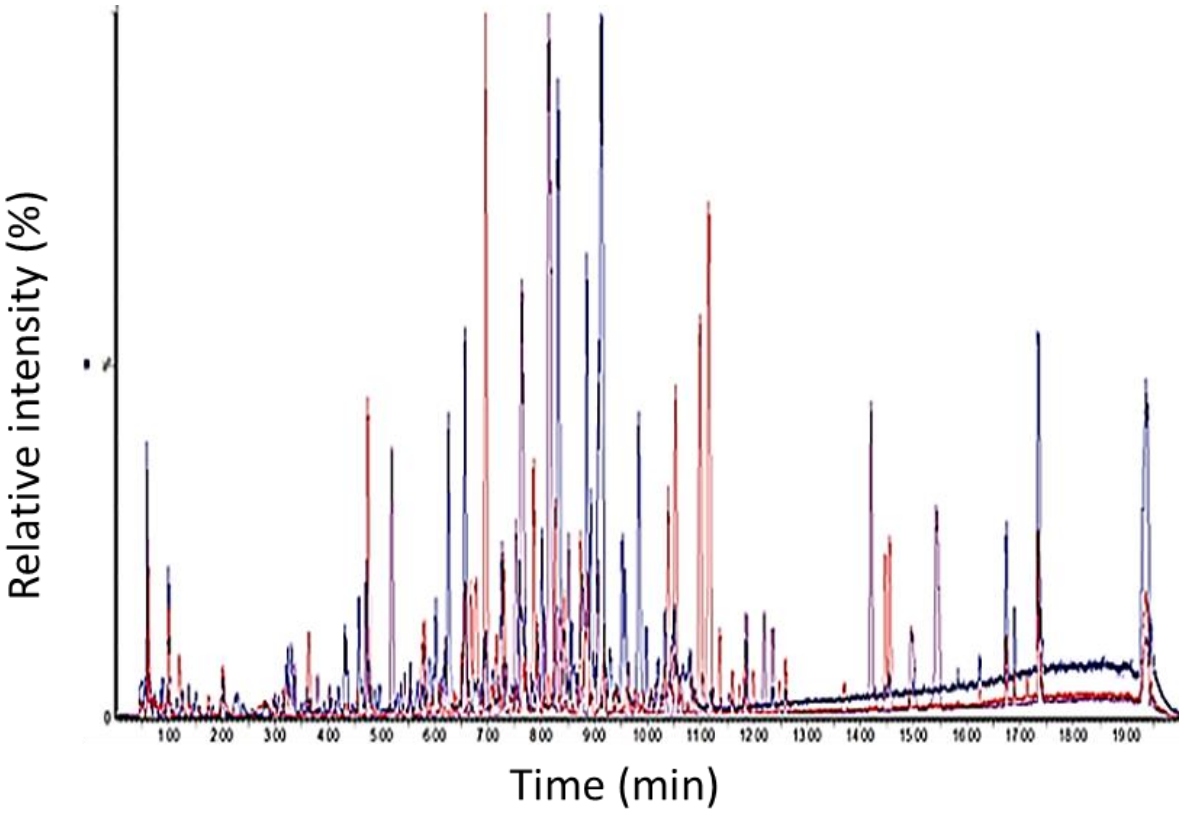

Figure 2.4. Overlay full scan chromatograms of oregano, olive leaves and myrtle leaves. Similar to the FT-IR spectra, there are clear chromatographic differences between the oregano and adulterant samples explaining the separation observed in the chemometric models (figure 2.3).

	Ionisation mode	Latent component	Orthogonal component	R2(cum)	Q2(cum)	RMSECV
Myrtle Vs Oregano	ESI -	1	1	0.994	0.984	0.062
	ESI +	1	3	0.999	0.994	0.039
Sumac Vs Oregano	ESI -	1	0	0.99	0.95	0.102
	ESI +	1	1	1	0.997	0.022
Olive Vs Oregano	ESI -	1	2	0.996	0.934	0.127
	ESI +	1	1	0.997	0.957	0.102
Cistus Vs Oregano	ESI -	1	2	0.997	0.982	0.066
	ESI +	1	5	1	0.961	0.097
Hazelnut Vs Oregano	ESI -	1	1	0.991	0.936	0.105
	ESI +	1	1	0.994	0.953	0.089

Table 2.1. Values of the statistical parameters obtained for different OPLS-DA models generated using UPLC-QTof MS data for both ionisation modes. R^2 (cumulative), Q^2 (cumulative) and Root Mean Squared Error of cross validation (RMSECV) were used to determine the validity of the models. R^2 (cum) indicates the variation described by all components in the model and Q^2 is a measure of how accurately the model can predict class membership.

Oregano Survey	UK/Ireland [a]	Internet/Other [b]
Samples Tested	53	25
Samples Adulterated	13	6
Samples Adulterated %	24.5	24
Level of Adulteration [c]	~30 to >70%	~30 to >70%
Most Common Adulterants	1. Olive leaves	1. Olive leaves
	2. Myrtle leaves	2. Myrtle leaves
[a] Includes Retail and Service Sector		
[b] Includes Amazon, Ebay and Purchases made abroad		
[c] Based on scores from chemometric analysis		

Table 2.2. Results from the oregano survey demonstrating that ~ 25% of the 78 commercial samples analysed were adulterated, most notably with olive and myrtle leaves. The levels of adulteration identified were ~30 to >70% indicating that significant amounts of commercial oregano adulteration are occurring.

2.6 References

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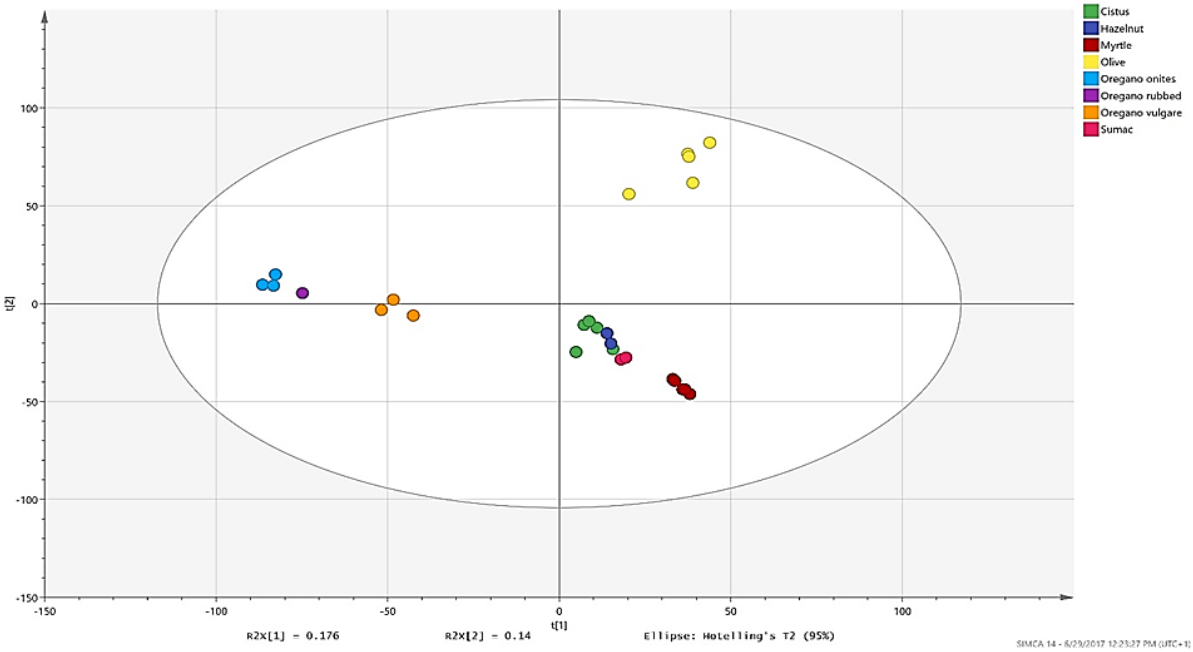
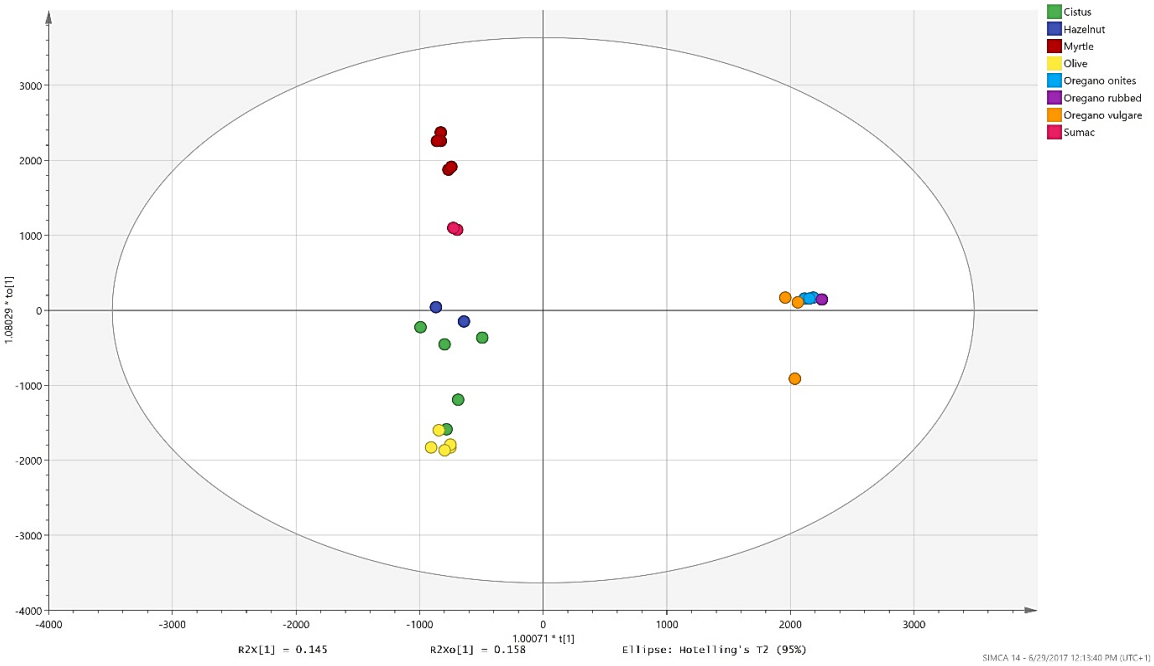
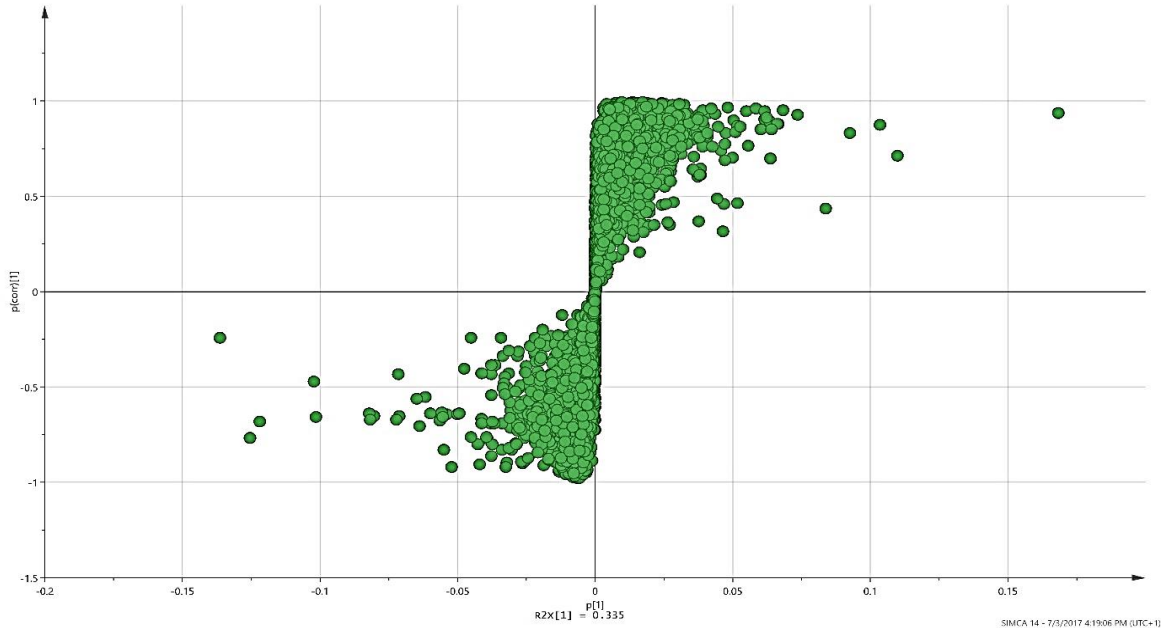
2.7 Appendix



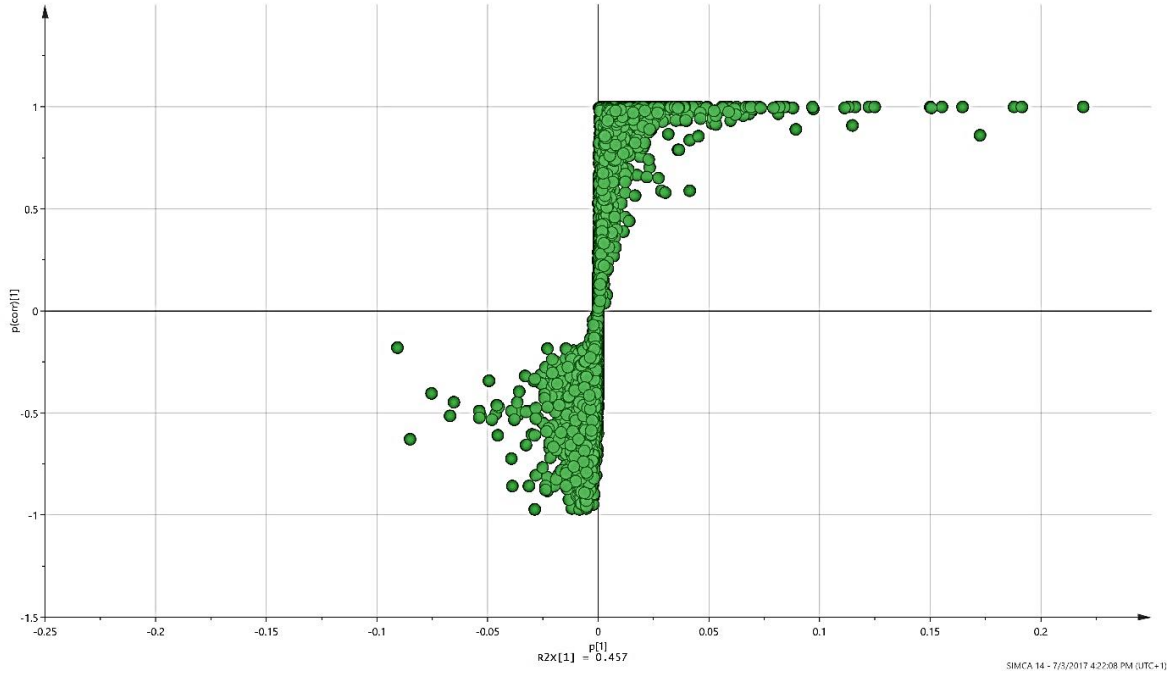
Unsupervised principal component analysis (PCA) score plot from liquid chromatography–high resolution mass spectrometry (LC-HRMS) data in positive ionisation mode, demonstrating clear separation between the oregano and adulterant samples.



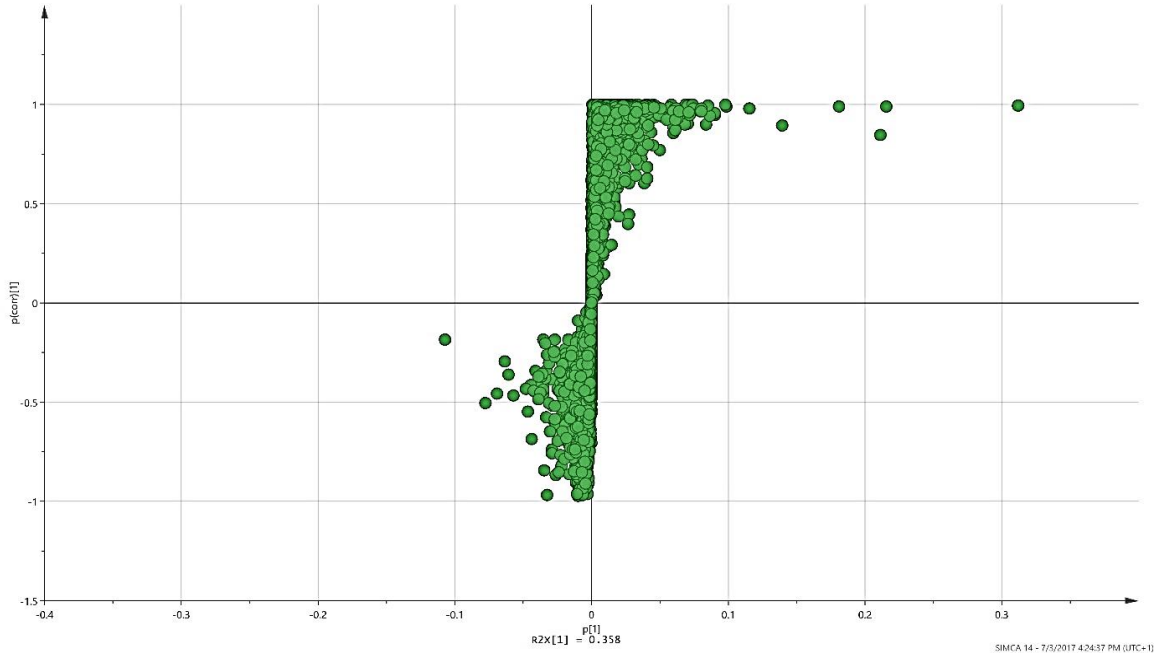
Supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) (1 latent and 3 orthogonal components) score plot from liquid chromatography–high resolution mass spectrometry (LC-HRMS) data in positive ionisation mode (R^2 = 99.7%; Q^2 = 94.4%).



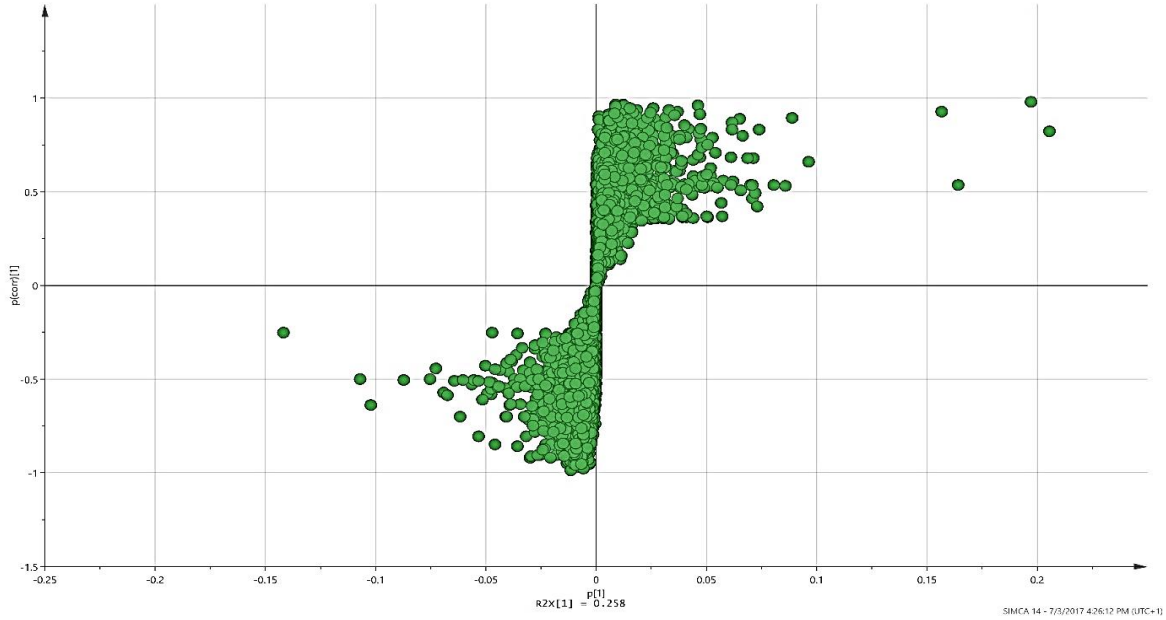
S-plot of olive leaves v oregano samples in positive ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



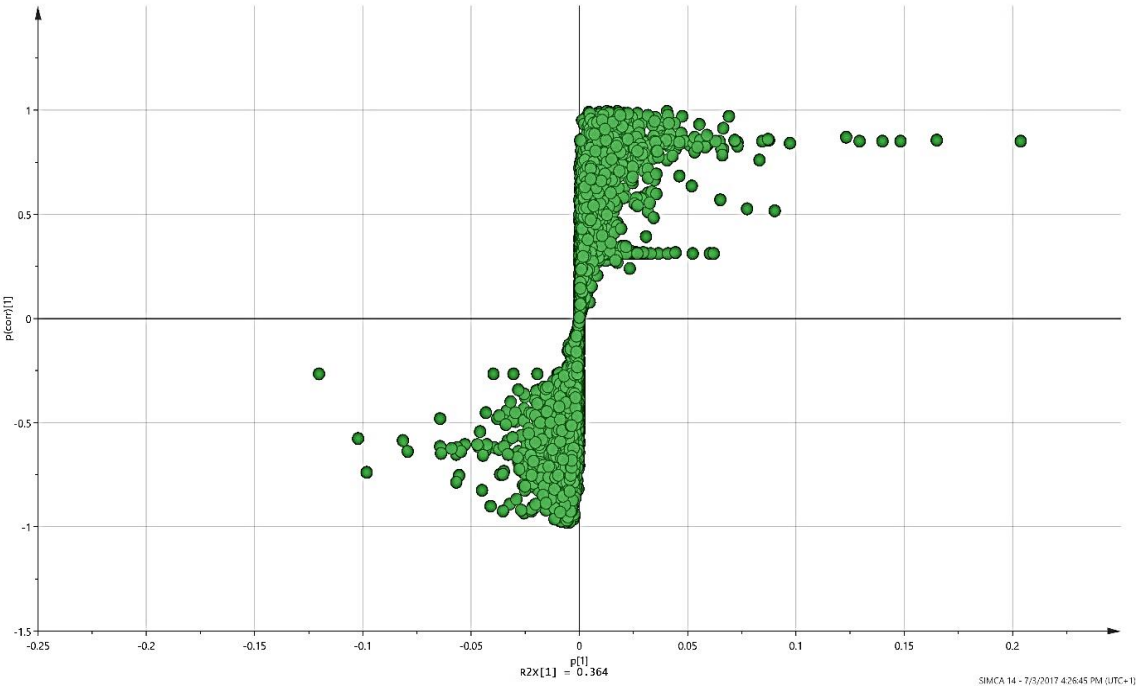
S-plot of sumac leaves v oregano samples in positive ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



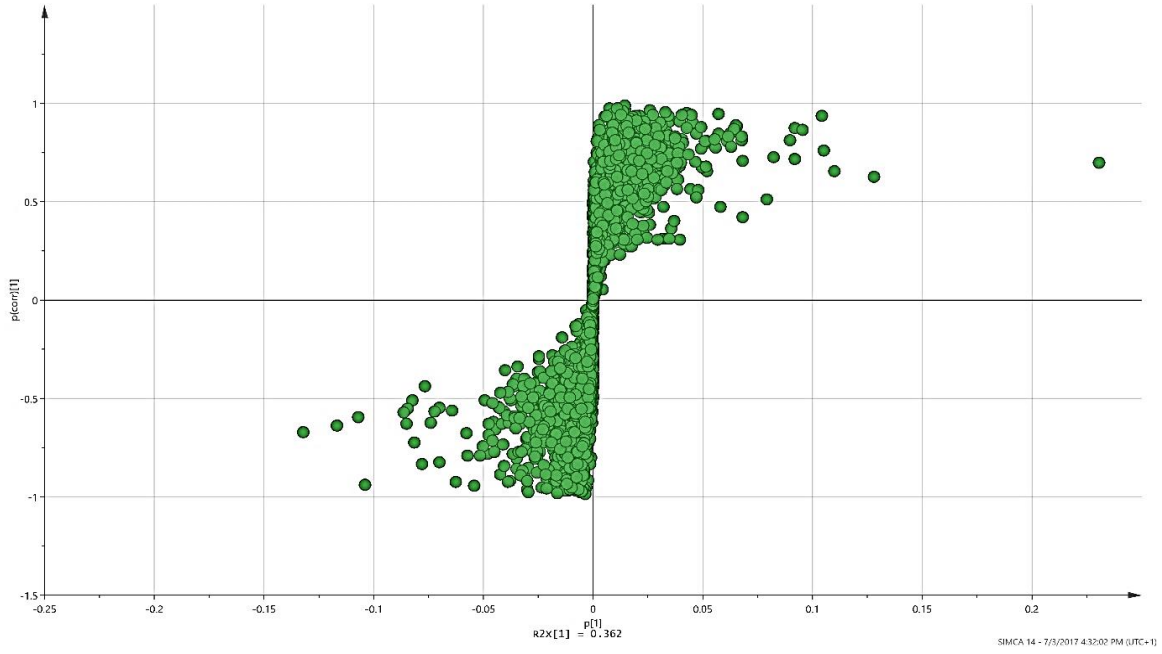
S-plot of hazelnut leaves v oregano samples in positive ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



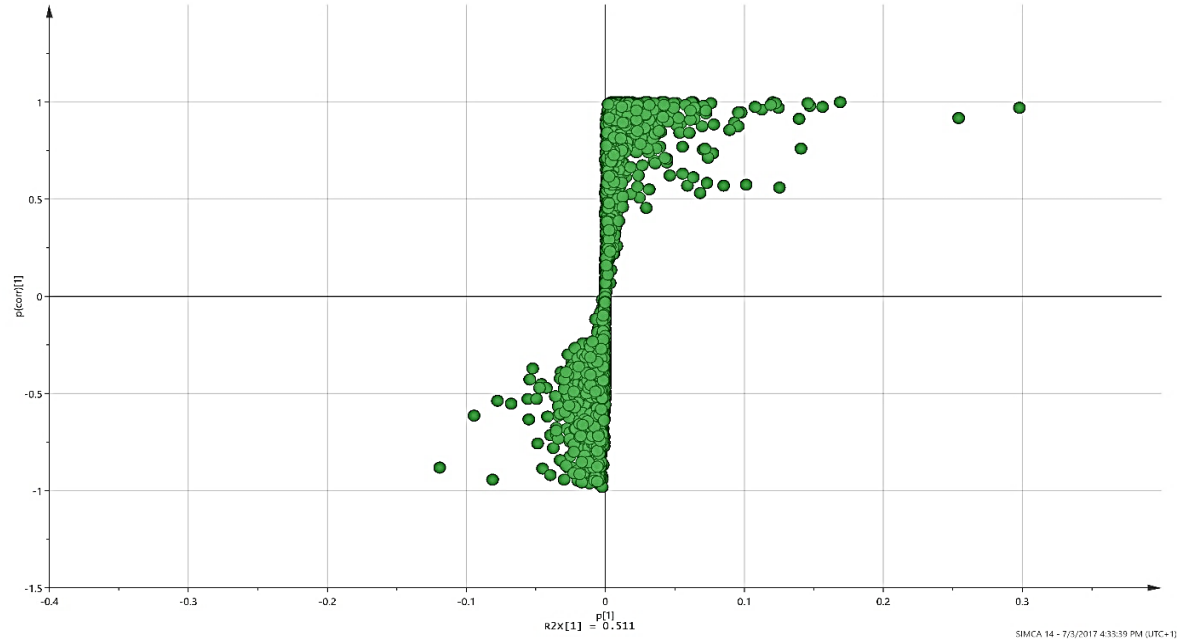
S-plot of cistus leaves v oregano samples in positive ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



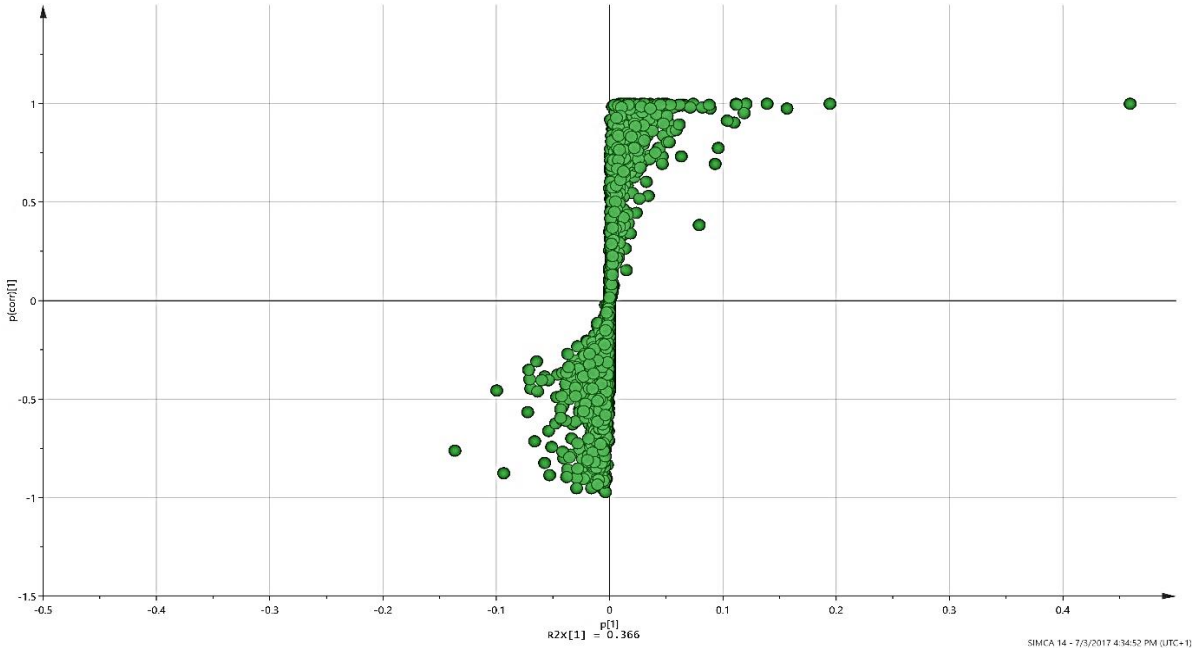
S-plot of myrtle leaves v oregano samples in positive ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



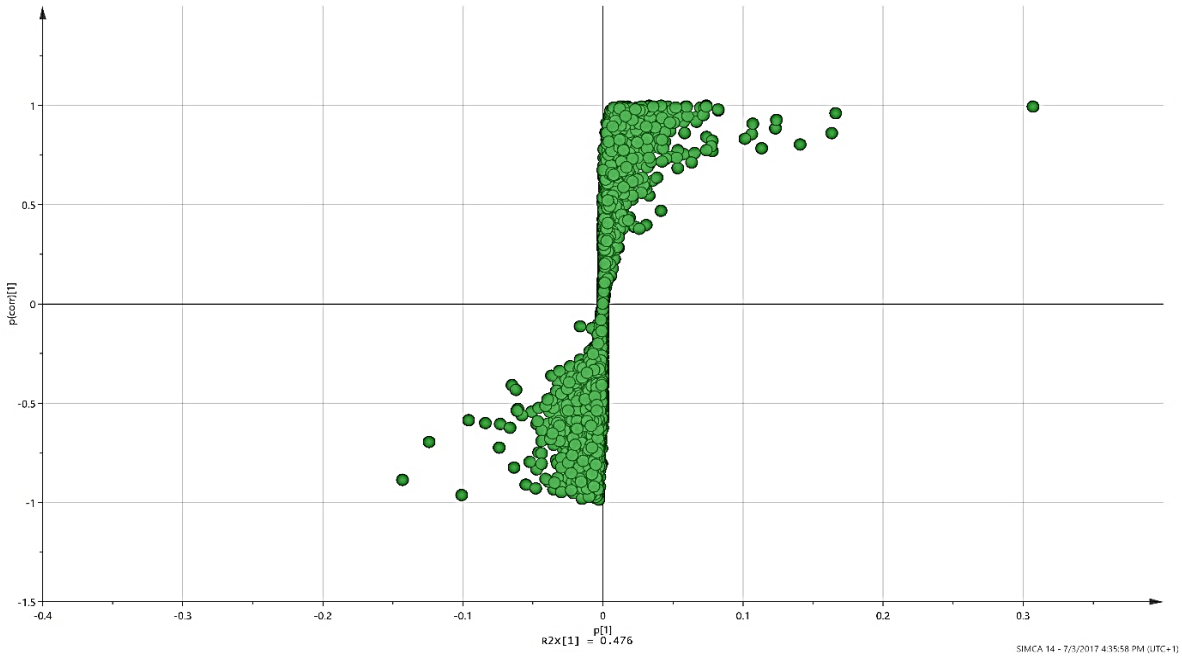
S-plot of olive leaves v oregano samples in negative ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



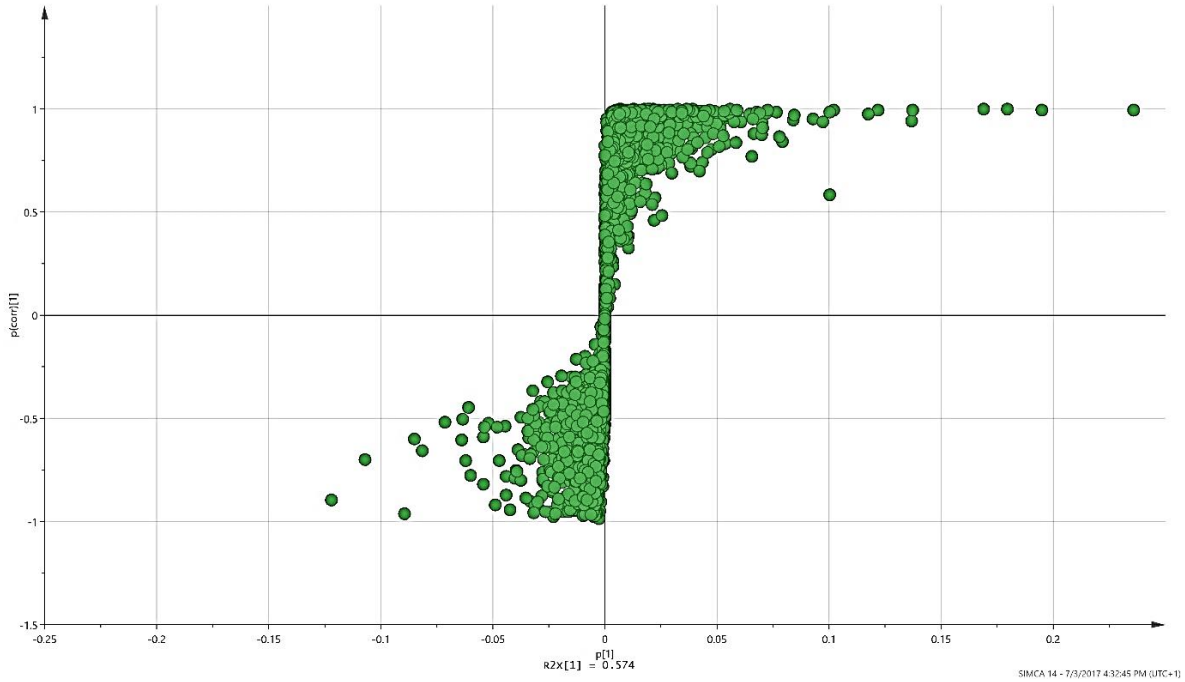
S-plot of sumac leaves v oregano samples in negative ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



S-plot of hazelnut leaves v oregano samples in negative ionisation mode identifying ions of significance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



S-plot of cistus leaves v oregano samples in negative ionisation mode identifying ions of importance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.



S-plot of myrtle leaves v oregano samples in negative ionisation mode identifying ions of importance (|p| > 0.03 and |p(corr)| >0.5) that contribute to the separation of the samples in the chemometric models.

Retention Time (min)	Molecular ion (m/z)	Sample reference
12.88	315.1	Cistus 1 pos
15.21	289.2	Cistus 2 pos
3.01	1101.1	Cistus 1 neg
4.53	1250.1	Cistus 2 neg
5.11	1250.1	Cistus 3 neg
9.61	297.1	Hazelnut 1 pos
8.94	297.1	Hazelnut 2 pos
8.93	475.2	Hazelnut 1 neg
11.21	763.3	Hazelnut 2 neg
9.15	555.2	Myrtle 1 pos
10.60	299.1	Myrtle 2 pos
8.47	381.1	Myrtle 1 neg
10.13	567.2	Myrtle 2 neg
2.21	268.1	Olive 1 pos
5.67	318.1	Olive 2 pos
6.51	151.0	Olive 3 pos
7.46	199.1	Olive 4 pos
7.55	151.0	Olive 5 pos
8.51	376.1	Olive 6 pos
9.81	379.1	Olive 7 pos
6.51	403.3	Olive 1 neg
12.02	827.4	Olive 2 neg
7.78	209.1	Sumac 1 pos
8.02	1075.1	Sumac 2 pos
11.34	539.1	Sumac 3 pos
7.78	1091.1	Sumac 1 neg
7.95	1091.1	Sumac 2 neg
11.34	537.1	Sumac 3 neg

List of ions identified for the adulterant samples (olive, sumac, hazelnut, myrtle and cistus leaves) using LC-HRMS in both positive and negative ionisation mode. Chemometric analysis and the generation of S-plots enabled the identification of the ions.

3. A real time metabolomic profiling approach to detect fish fraud using rapid evaporative ionisation mass spectrometry

3.1 Abstract

Fish fraud detection is mainly carried out using a genomic profiling approach requiring long and complex sample preparations and assay running times. Rapid evaporative ionisation mass spectrometry (REIMS) can circumvent these issues without sacrificing a loss in the quality of results. To demonstrate that REIMS can be used as a fast profiling technique capable of achieving accurate species identification without the need for any sample preparation. Additionally, we wanted to demonstrate that other aspects of fish fraud other than speciation are detectable using REIMS. 478 samples of five different white fish species were subjected to REIMS analysis using an electrosurgical knife. Each sample was cut 8-12 times with each one lasting 3-5 seconds and chemometric models were generated based on the mass range m/z 600-950 of each sample. The identification of 99 validation samples provided a 98.99% correct classification in which species identification was obtained near-instantaneously (≈2s) unlike any other form of food fraud analysis. Significant time comparisons between REIMS and polymerase chain reaction (PCR) were observed when analysing 6 mislabelled samples demonstrating how REIMS can be used as a complimentary technique to detect fish fraud. Additionally, we have demonstrated that the catch method of fish products is capable of detection using REIMS, a concept never previously reported. REIMS has been proven to be an innovative technique to aid the detection of fish fraud and has the potential to be utilised by fisheries to conduct their own quality control (QC) checks for fast accurate results.

Keywords – REIMS; real time; no sample preparation; fish; species identification; catch method

3.2 Introduction

Economically motivated adulteration (EMA) of seafood products is a global issue occurring at alarmingly high rates (table 3.1) with it estimated that on average 30% of commercial fish products sold are either misrepresented or mislabelled.[1] This equates to fraud in almost $120 billion of the global seafood industry as the Food and Agriculture Organisation of the United Nations (FAO) estimate the global seafood industry to be worth $400 billion annually, with global industry analysts expecting this value to rise to $430 billion by 2018.[2]

Genomics, proteomics, metabolomics and lipidomics are four alternative and in some cases complimentary systems biological approaches often employed for food fraud detection studies.[3] Most fish fraud detection studies utilise genomic profiling as DNA is found in all cells and organisms and can be analysed in all types of tissue ranging from freshly caught fish to processed and cooked samples.[4] Whilst very accurate qualitative and quantitative results are achievable using polymerase chain reaction (PCR), it comes at the expense of long and often complex sample preparations coupled with long assay running times. In terms of managing fraud in fast moving supply chains this is a substantial disadvantage.

Ambient mass spectrometry (AMS) is a relatively new field of analytical chemistry which is showing promise at detecting food fraud.[5] The recent increase in popularity of these techniques is a result of minimal or no sample preparation being required and fast assay running times. Whilst excellent qualitative results are achievable, it would appear quantitatively they struggle, especially with solid samples.[5,6] Whereas some food commodities such as meat,[7] dairy products,[8] olive oil [9] and spices [10] have been subjected to analysis using AMS techniques, fish has yet to receive the same level of investigation.

Rapid evaporative ionisation mass spectrometry (REIMS) is one of the newest forms of AMS and, as is the case with many analytical innovations was created for medical research purposes. It operates using an electrosurgical knife, bipolar forceps or laser which creates an aerosol (smoke) when cutting into a tissue sample. The aerosol is evacuated from the sample through a transfer line into the ionisation source of a mass spectrometer where a heated collision

surface is situated and the ionisation process occurs. Although the majority of publications utilising REIMS have centred on medical (tissue identification) and bacterial identification applications,[11,12] there are early indications that it may also find applications in the detection of food fraud.[13] Results are obtained near-instantaneously (2-3 seconds) and the technique appears to be able to achieve semi-quantitative results for solid samples without the need for any form of sample preparation within a liquid solution.

In the present study, REIMS was applied to five commercially popular and genetically similar white fish species (cod, coley, haddock, pollock and whiting) and investigated as to whether fast and accurate speciation results could be obtained. The REIMS technology was believed to have the capability to determine the sample species in real time, unlike most forms of analytical systems employed for such studies. Additionally, this study demonstrates the possibility of distinguishing between different catch methods within a species, an aspect of fish fraud which is well known but has never been previously reported.

3.3 Methods

3.3.1 Sampling

This study was based upon five commercially popular white fish species. All tissue samples (fillets, tails and unspecified areas) of cod, coley, haddock, pollock and whiting were sourced from trusted suppliers and stored at -80^{o}C. Samples of seabass and seabream fillets were sourced from Italy and stored at -80^{o}C. Prior to REIMS analysis the samples were thawed at room temperature for two hours in the fumehood where the REIMS cutting took place.

3.3.2 REIMS experimental setup

The experimental setup for this study was similar to that reported previously.[13] A Medimass REIMS source (Medimass, Budapest, Hungary) was mounted orthogonally to the interface of a Xevo G2-XS quadrupole time-of-flight (QTof) mass spectrometer (Waters Corporation., Wilmslow, UK) which was operated in negative ion and sensitivity mode. Mass spectra data were acquired over the range m/z 200-1200 with a scan time of 0.5s. The REIMS source was connected to a monopolar electrosurgical knife (Model PS01-63H, Hangzhou medstar

technology Co, Ltd, Jiaxing City, China) through a 3m long, 1cm diameter ultra-flexible tubing (evacuation/vent line). Electrosurgical dissection in all experiments was performed using an Erbe VIO 50 C generator (Erbe Medical UK Ltd, Leeds, UK). The generator was operated in 'autocut' mode with a power setting of 30W. All samples were cut on the return electrode and a venturi gas jet pump driven by nitrogen (1 bar) evacuated the aerosol produced at the sample site towards a heated kanthal coil that was operated at 6.4W (2.8A @ 2.3V). A lockmass solution of Leucine Enkephalin (LeuEnk) (m/z 554.2615) (2ng / µL) in isopropanol (IPA) was infused using a Waters Acquity UPLC I-class system (Waters Corporation., Milford, MA, USA) at a continuous flow rate of 0.1 mL/min for accurate mass correction. Prior to analysis the mass spectrometer was calibrated using 5mM sodium formate solution (90% IPA) at a flow rate of 0.2 mL/min for two minutes. Dependent on the size, each tissue sample was cut 8-12 times for reproducibility with each cut lasting approximately 3-5s. This enabled multiple locations on each tissue sample to be analysed. The delay between sampling and appearance of a signal was ≈2s, with no carry-over effects visible between each burn and/or sample.

3.3.3 REIMS data pre-processing and analysis.

Principal component analysis (PCA), an unsupervised technique, linear discriminant analysis (LDA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), both supervised techniques, were used to build the qualitative speciation and catch method models within this study.

Raw data generated by the mass spectrometer were pre-processed using a prototype software (Waters Research Centre, Budapest, Hungary) that used standard Masslynx pre-processing algorithms (Waters). The recorded scans for each sample were combined to give an average spectrum and thus one spectrum for each sample was used to build the chemometric models. The resulting data were lockmass corrected using LeuEnk (m/z 554.2615) and normalised (Total Ion Count - TIC) before being exposed to multivariate analysis. All chemometric models were calculated using the mass region of m/z 600-950, a spectral intensity threshold

of $2e^6$ counts and a bin width of 0.5 Da. When using a m/z range for models that included LeuEnk, variations in the lockmass intensity and interferences with the lockmass compound resulted in a degree of irreproducibility/error. PCA was used to reduce the dimensionality of the data prior to LDA analysis using the first 25 PCA components. The prototype software enabled a leave-20%-out cross-validation of the PCA-LDA score plots in which one average spectrum per sample was analysed. A model was calculated using 80% of the samples and data files left out were classified using the training model. This was repeated five times enabling each sample to be left out once from the model building process. Using a standard deviation of 5σ, each sample was classified to the closest class. If a sample was outside the standard deviation range of 5σ for all classes, then it was marked as an outlier.

The processed matrix generated within the prototype modelling software was exported to SIMCA 14 (Umetrics, Umea, Sweden) allowing the data to be exposed to further chemometric functions such as OPLS-DA. All data was mean-centred, pareto scaled and grouped accordingly into the five species of fish. R^2 (cumulative), Q^2 (cumulative) and a misclassification table were used to determine the validity of the models. R^2 (cum) indicates the variation described by all components in the model and Q^2 (cum) is a measure of how accurately the model can predict class membership. Permutation tests were carried out to ensure the models were not over-fitted. Individual OPLS-DA speciation models and S-plots of each species of fish against the other four species were generated to identify ions of significance for each species.

3.3.4 Real time recognition of samples

The PCA-LDA models created using the prototype software were exported to a prototype recognition software (Waters Research Centre, Budapest, Hungary) allowing for real-time identification of samples. Raw data files were acquired and ran live though the software providing a near-instantaneous identification, excluding the delay between sampling and appearance of a signal which was ≈2s. A standard deviation of 5σ was used for class

assignment. The spectral intensity limit was set at $1e^8$ counts thus ensuring that only the cuts were assigned a species classification and not any background noise.

3.3.5 DNA analysis setup and analysis

Mitochondrial cytochrome c oxidase subunit I gene (*COI*) was used as genetic marker for the examination of samples. DNA extraction was performed using a commercial kit (NucleoSpin Tissue – Macherey Nagel) according to the manufacturer guidelines. A fragment of approximately 655bp of *COI* was amplified using the primer pair COIfish_F1 (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and COIfish_R1 (5'-ACTTCYGGGTGRCCRAARAATCA-3') in a PCR reaction.[14] The sequences were determined by direct DNA sequencing on both strands of the PCR products by BigDye Terminator v3.1 cycle sequencing kit using the amplification primer pair and analysed on ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Sequences were compared with those deposited in GenBank and Barcode of Life Data Systems (BOLD). Results were considered valid above 98% of similarity.

3.4 Results

3.4.1 REIMS fish speciation

Raw spectrometric data (supplementary information S1) obtained from authenticated samples of cod (n=194), coley (n=51), haddock (n=133), pollock (n=50) and whiting (n=50) were pre-processed and subjected to multivariate analysis where PCA, LDA and OPLS-DA were applied. 80 PCA components and 4 LDA components were used to generate the chemometric models. Clustering was identified within the three-dimensional (3-D) PCA score plot using components 1,2 and 4 (figure 3.1a). However, clear separation between the five species of fish was obtained within the 3-D LDA score plot using components 1,2 and 4 (figure 3.1b) and the OPLS-DA score plot where 4 latent and 4 orthogonal components were used (figure 3.1c). A leave-20%-out cross-validation of the PCA-LDA models, where one average spectrum per sample was used resulted in a 99.37% correct classification (supplementary information S2) which was due to two samples being assigned an outlier classification and one whiting being

identified as coley. Additionally, a correct classification rate of 99.37% was obtained for the OPLS-DA model (supplementary information S3) which was due to two cod samples being identified as coley and whiting, and one coley sample being identified as whiting. R^2 and Q^2 values of 0.829 and 0.809 indicated that the OPLS-DA model had both a good quality of fit and predictivity towards new data. A large Q^2 value also suggests that the multivariate data points are well clustered with there being very few outliers within the dataset as exemplified in all the chemometric models within figure 3.1. The relevant permutation tests (supplementary information S4) were carried out to demonstrate that the models were not over-fitted.

3.4.2 Real time validation of speciation model

Raw spectrometric data obtained from authenticated samples of cod (n=22), coley (n=20), haddock (n=20), pollock (n=20) and whiting (n=17), which had not been previously used to generate the chemometric models were run live through the prototype recognition software providing a near-instantaneous (≈2s) identification (figure 3.2). Of the 99 samples analysed, 98 (98.99%) were correctly identified with one cod sample being assigned as an outlier (unidentified).

3.4.3 Statistical validation of speciation model

The second approach of the validation was carried out to ensure the validity of the results from the prototype recognition software. The raw data acquired from the 99 samples were subjected to a cross-validation similar to that of the leave-20%-out cross-validation. A model was created using the training set of samples used to generate the speciation models (n=478) excluding the 99 validation samples. Each validation sample was then assigned a fish species classification using one average spectrum and a standard deviation of 5σ. The results were in agreement to that of the recognition software and a correct classification rate of 98.99% was obtained (supplementary S5).

3.4.4 DNA analysis of suspect 'haddock' samples

During the investigation and generation of the speciation models it was found that six samples labelled as 'haddock' were clustered within the cod samples in all chemometric models. Additionally, the prototype recognition software identified all six 'haddock' samples as cod in which it took 15/20 minutes to obtain results for all the samples. As a result, the samples were further analysed using PCR to establish whether they were indeed haddock or whether they had accidentally been mislabelled. Mitochondrial cytochrome c oxidase subunit I gene (*COI*) was used as genetic marker for the six samples, in which all showed 99% similarity with *Gadus morhua* species (cod) on both Genbank and BOLD. No significant similarities were observed with *Melanogrammus aeglefinus* (haddock).

3.4.5 Real time analysis of seabass and seabream samples

Raw spectrometric data obtained from authenticated samples of seabass (n=6) and seabream (n=8) were simultaneously run live through the prototype recognition software providing a near-instantaneous (≈2s) classification. Of the 14 samples analysed, 13 (92.86%) were correctly identified as outliers with one sample being identified as both an outlier (66%) and coley (34%) sample.

3.4.6 Statistical validation of seabass and seabream samples

The second approach of the validation was carried out to ensure the validity of the results from the prototype recognition software. The raw data acquired from the 14 samples were subjected to a cross-validation like that of the leave-20%-out cross-validation. A model was created using the training set of samples used to generate the speciation models (n=478) excluding the 14 seabass and seabream samples. Each sample was then assigned a fish species classification using one average spectrum and a standard deviation of 5σ. An overall correct classification rate of 100% for all 14 samples was obtained as the cross-validation uses a single averaged spectrum of all the cuts per sample resulting in the one seabream sample which was assigned as both an outlier (66%) and coley (34%) sample being assigned an outlier classification.

3.4.7 Catch method of haddock

Raw spectrometric data obtained from both line caught (n=35) and trawl caught (n=65) haddock samples were exposed to multivariate analysis allowing PCA, LDA and OPLS-DA models to be generated. 20 PCA components and 2 LDA components were used to generate the catch method models. Some separation was apparent within the 3-D PCA score plot using components 1,2 and 3 (figure 3.3a). However, clear separation was attained in the two-dimensional (2-D) LDA score plot using components 1 and 2 (figure 3.3b), and the OPLS-DA score plot (figure 3.3c) in which 1 latent and 3 orthogonal components were used. A leave-20%-out cross-validation of the PCA-LDA models resulted in a 95.00% correct classification with three trawl caught and two line caught samples being misidentified (supplementary information S6). However, a correct classification rate of 100% was obtained for the OPLS-DA model. R^2 and Q^2 values of 0.863 and 0.746 were obtained suggesting that the OPLS-DA model was both robust and had good predictability towards a new set of data. The relevant permutation tests (supplementary information S7) were carried out to demonstrate that the models were not over-fitted.

3.5 Discussion

Industries across the food sector want fast and accurate results when undertaking their own quality control (QC) checks. DNA approaches, of which most of the studies in table 3.1 have employed, fulfil the criteria of obtaining accurate results, but it comes at the expense of long sample preparations and assay running times. Validation of the chemometric speciation models, in which a 98.99% correct classification (table 3.2) was achieved using the prototype recognition software (figure 3.2) clearly shows that REIMS can fulfil the principle of real time profiling without sacrificing the quality of results that are obtained. Considering that no sample preparation is required, which is a major pitfall for PCR, it is evident that REIMS and maybe other AMS techniques have a prominent role to play in tackling fish fraud.[15] As each sample is cut 8-12 times it could be possible that the raw data acquired using REIMS is analogous to that of liquid chromatography-mass spectrometry (LC-MS), a ‘classical’

technique often used when carrying out metabolomic profiling experiments. Perhaps from an analytical variability standpoint (QC pooled samples) LC-MS is more suited towards such metabolomic profiling experiments.[16] But, in a real-world situation where species identification is both desired and needed rapidly (fishery, port loading dock, etc.) LC-MS cannot compete with the REIMS technology.

The mislabelling of the six 'haddock' samples signifies the vast time comparisons that exist between PCR and REIMS. Whereas the REIMS technology in conjunction with the prototype recognition software provided a result for each sample burn within seconds (including sample preparation), PCR analysis of the six samples took 24 hours, including time taken for sample preparation. Both analytical platforms produced identical results and it is evident that REIMS has the capability to analyse many samples within the timeframe taken for a PCR result. These time-based comparisons are very significant as it demonstrates how companies with fast moving supply chains could be operating their own QC checks in the future, with fast and accurate results attainable within seconds which is ultimately what they desire.

Fast results are coveted but not at the expense of false positive and negative identifications. The versatility of the REIMS and strength of the chemometric models, evaluated by R^2 = 0.829, Q^2 = 0.809 and the permutation tests (supplementary information S4), is also demonstrated by the 8 seabream and 6 seabass samples. All 14 samples were correctly identified as outliers with one seabream sample being assigned both an outlier (66%) and coley (34%) sample. However, because a greater majority of the cuts were identified as an outlier and not coley, the statistical validation of all 14 samples gave a 100% correct classification as the software uses one average spectrum of all the cuts for each sample. Along with the validation of the speciation models and the PCR testing of the six suspect 'haddock' samples, the classification of the 14 seabass and seabream samples as outliers further illustrates that fish speciation is very achievable using REIMS with fast and accurate results attainable. Compared to PCR, the coupling of the REIMS source to a XEVO G2-XS QTof mass spectrometer does result in large cost differences. However, in this study only a few aspects of the QTof mass spectrometer were utilised; the time-of-flight (Tof) tube and the detector.

MS/MS functions such as the quadrupole and collision induced dissociation (CID) were not and therefore, it may be possible to couple the REIMS source to a cheaper and perhaps smaller alternative as the development of miniaturised and fieldable mass spectrometers appears to be making significant advances.[17] Paper spray (PS), desorption atmospheric pressure chemical ionisation (DAPCI) and several other AMS plasma based sources (dielectric barrier discharge ionisation (DBDI), low temperature plasma ionisation (LTP) and plasma-assisted desorption ionisation (PADI)) have reportedly been coupled to a miniature mass spectrometer instrument.[17] However, in practice it will be a long time until the use of miniaturised mass spectrometers becomes common practice.

The aim of this study was to demonstrate that REIMS can be used as a fast profiling technique which the fish and perhaps the whole food industry can use to carry out QC checks and that there are significant time comparisons that exist between REIMS and techniques that are commonly associated with such studies like PCR and LC-MS. Yet, within the study it has been found that there are potential ions of significance for pollock (figure 3.4 (a-c)) and the other four species of fish (supplementary S8-S9). The significance of the chosen ions was exhibited by their variable importance in projection (VIP) values ($x > 1$), their S-plot $|p|$ values ($x > 0.03$) and their S-plot $|p(corr)|$ values ($x > 0.5$). Putative identifications were assigned by carrying out a targeted MS/MS approach which involved collision induced dissociation (CID) to obtain fragments for the three pollock ions identified in figure 3.4 and the ion thought to have the greatest influence towards the separation of the other four species of fish within the chemometric models. Based on previous studies carried out using the REIMS technology and the mass range that we have utilised to generate the chemometric models, we expected the ions to be phospholipids.[11,18] Putative identifications could not be assigned to every ion but the fragments identified in table 3.2 suggest a mixture of isobaric and isomeric phospholipid species and/or the presence of other lipid species. For the ions of which it was possible to assign a classification, it is believed that they are most likely to be one of two different classes of phospholipid; phosphatidylethanolamine (PE) and phosphatidylserine (PS). Multiple lipid classes have been assigned due to the lack of chromatographic separation that accompanies

REIMS analysis. The only ion not to be identified as a phospholipid species was m/z 655.5 $[2M-H]^-$ which is believed to be a dimer of docosahexaenoic acid (DHA) (m/z 327.21 $[M-H]^-$). Fragment ions of m/z 283.25 suggest loss of CO_2 from DHA and m/z 229.20 suggests a McLafferty rearrangement.

Substitution of one species of fish for another is by far the most commonly reported with regards to fish fraud. However, there are six other forms in which it can manifest itself; IUU fishing; fishery substitution; processed raw material authenticity (species adulteration); chain of custody abuse; undeclared product content and catch method.[19] To date, the scientific investigation of different catch methods within the same species of fish has never been reported. Separation of the two haddock catch methods was achieved (figure 3.3 (a-c)) but it is unclear as to whether this was due to genuine differences in which way the fish samples were caught. REIMS spectral data are thought to be dominated by intact phospholipids and fatty acids. However, differences in the catch method of a fish would not be thought to affect the lipid profile of a fish unless they had different diets which may be a result of line caught fish being caught at shallower depths compared to that of trawl caught samples. A more plausible explanation is that the two different catch methods are likely to affect secondary metabolites (stress markers) within a fish sample. Compared to speciation, multivariate analysis of the catch method data did not result in any reliable ions that could explain separation within the models. The two ions believed to provide the greatest variance between the two catch methods, according to the S-plot, were m/z 764.5 and m/z 819.5 with the former thought to occur at more abundant levels in trawl caught samples and the latter in line caught samples. Similar to the speciation results, it is expected that numerous isobaric and isomeric lipid species are assignable to the two masses due to the lack of chromatographic separation that occurs within REIMS analysis. A search of known stress markers did not result in any assignments either. A larger study with equal amounts of samples for each class is required to confirm this. However, whichever scenario it may be, separation between the two catch methods has been achieved and therefore, this is the first scientific study to demonstrate that differentiating between line and trawl caught samples within the same species is possible.

3.6 Conclusions

No sample preparation, accurate and near-instantaneous results are three properties which the REIMS technology has exemplified in this study and are all three issues which cannot be fulfilled by most analytical platforms used for such fish studies. The large time comparisons (15/20 mins – 24 hours) observed between REIMS and PCR to determine the species of six mislabelled samples are hugely significant. REIMS is a frontier technology not found in common analytical laboratories but it is clear that it has the potential to be utilised in commercial environments. In the short run, it could be seen as a complimentary, albeit expensive technique to help aid the detection of commercial fish fraud whilst in the long run a miniaturised and cheaper version of the technology could be utilised by fisheries to conduct their own QC checks. As well as this, REIMS has shown to be able to analyse multiple aspects of fish fraud through the separation of line and trawl caught haddock samples and it may well be that there are other aspects such as geographic origin and wild/farmed which can be differentiated, further issues which genomic profiling is ill-equipped to do.

Acknowledgements

We would like to thank the BBSRC (BB/M0503162/1) and Waters Corporation for their funding and continuous support throughout this work. We would also like to acknowledge Young's seafood limited for supplying authenticated fish samples as well as Wm. Morrison Supermarkets plc for supplying authenticated haddock samples for validation purposes.

Country	Number of samples analysed	Mislabelling rates (%)	Reference
Australia	38	0	*20*
Brazil	30	24	*21*
Canada	236	41	*22*
China	42	86	*23*
Egypt	90	33	*24*
France	371	3.7	*25*
Germany	145	6.2	*26*
India	100	22	*27*
Iran	27	11	*28*
Italy	69	32	*29*
Japan	26	8	*30*
Malaysia	62	16	*31*
Portugal	178	6.7	*26*
Republic of Ireland	131	28	*32*
South Africa	149	18	*33*
Spain	245	7.8	*34*
Turkey	50	86	*35*
USA	216	13	*36*
United Kingdom (UK)	386	5.7	*37*

Table 3.1. Global studies aimed at investigating the mislabelling rates of fish samples.

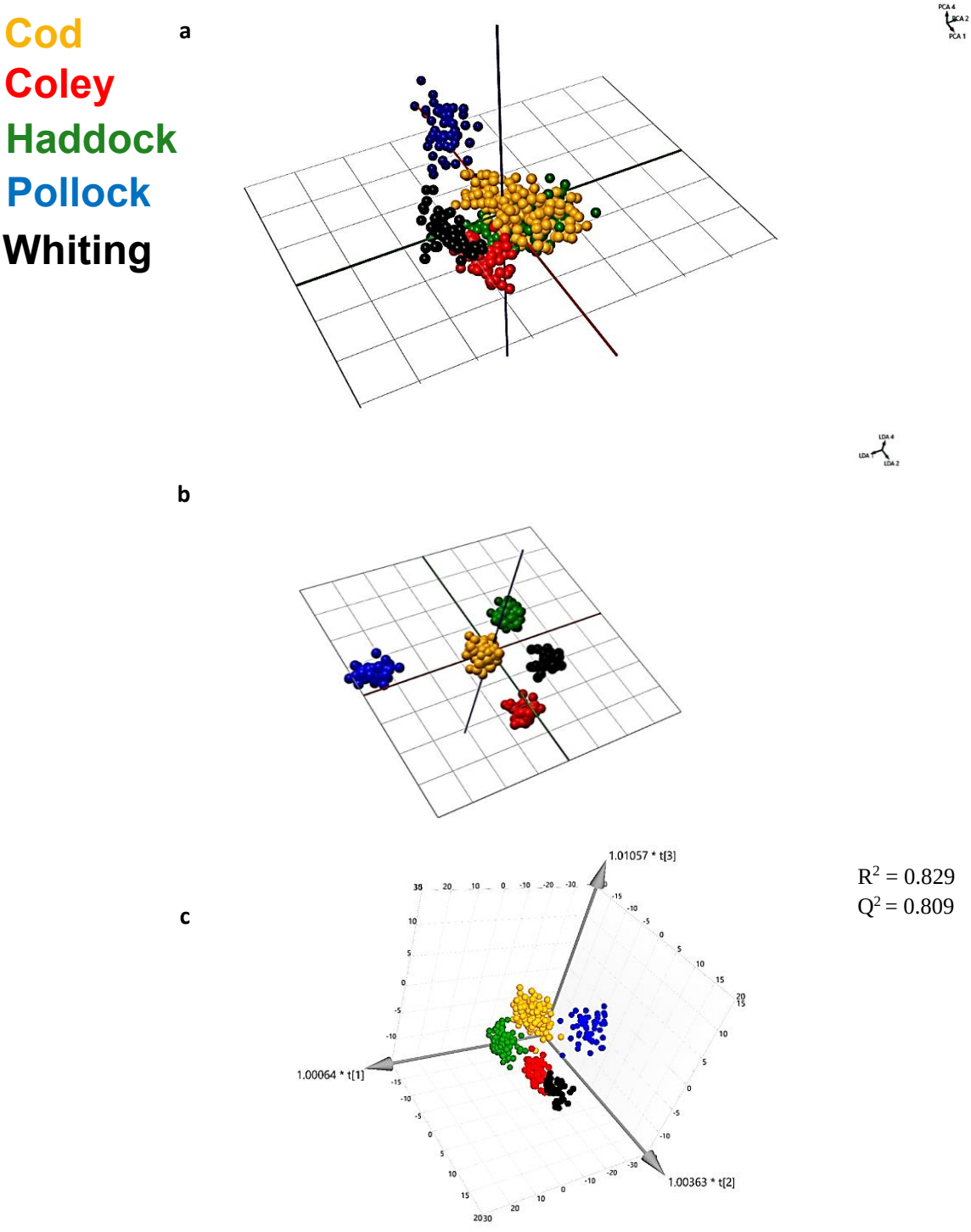


Figure 3.1. (a) Principal component analysis (PCA) (80 PCA components), (b) linear discriminant analysis (LDA) (4 LDA components) and (c) orthogonal partial least squares-discriminant analysis (OPLS-DA) (4 latent and 4 orthogonal components) models generated using the prototype software and SIMCA 14. All models were generated using a bin of 0.5Da and the mass range m/z 600-950 of the fish samples with clear separation of the five-fish species of fish; cod (orange), coley (red), haddock (green), pollock (blue) and whiting (black) visible within the 3-D LDA and OPLS-DA models.

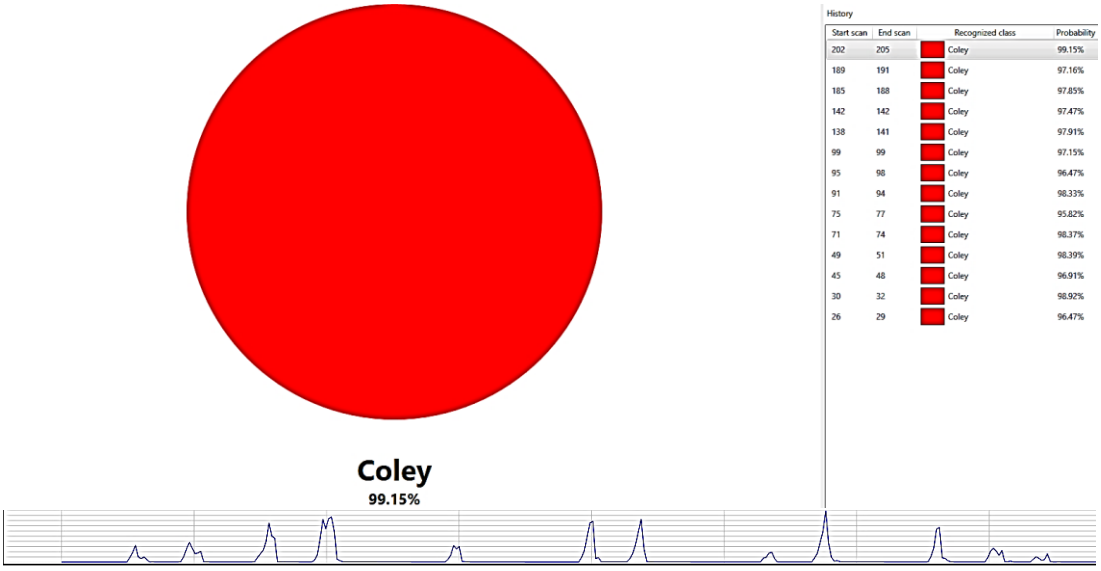


Figure 3.2. Validation of the speciation models using the prototype recognition software and a further set of authenticated fish samples. In this scenario, the sample under investigation is coley and the figure above demonstrates the recognition software correctly identifying a sample burn to be coley (red circle). The results for each burn are obtained near-instantaneously excluding the delay between sampling and appearance of a signal which was ≈2s. Twelve cuts were taken from this sample which is identified in the chromatogram with identification for some of the cuts identified on the right-hand side of the figure. A standard deviation of 5σ was used for class assignment. Of the 99 samples analysed, 98 (98.99%) were correctly identified with one cod sample being assigned as an outlier.

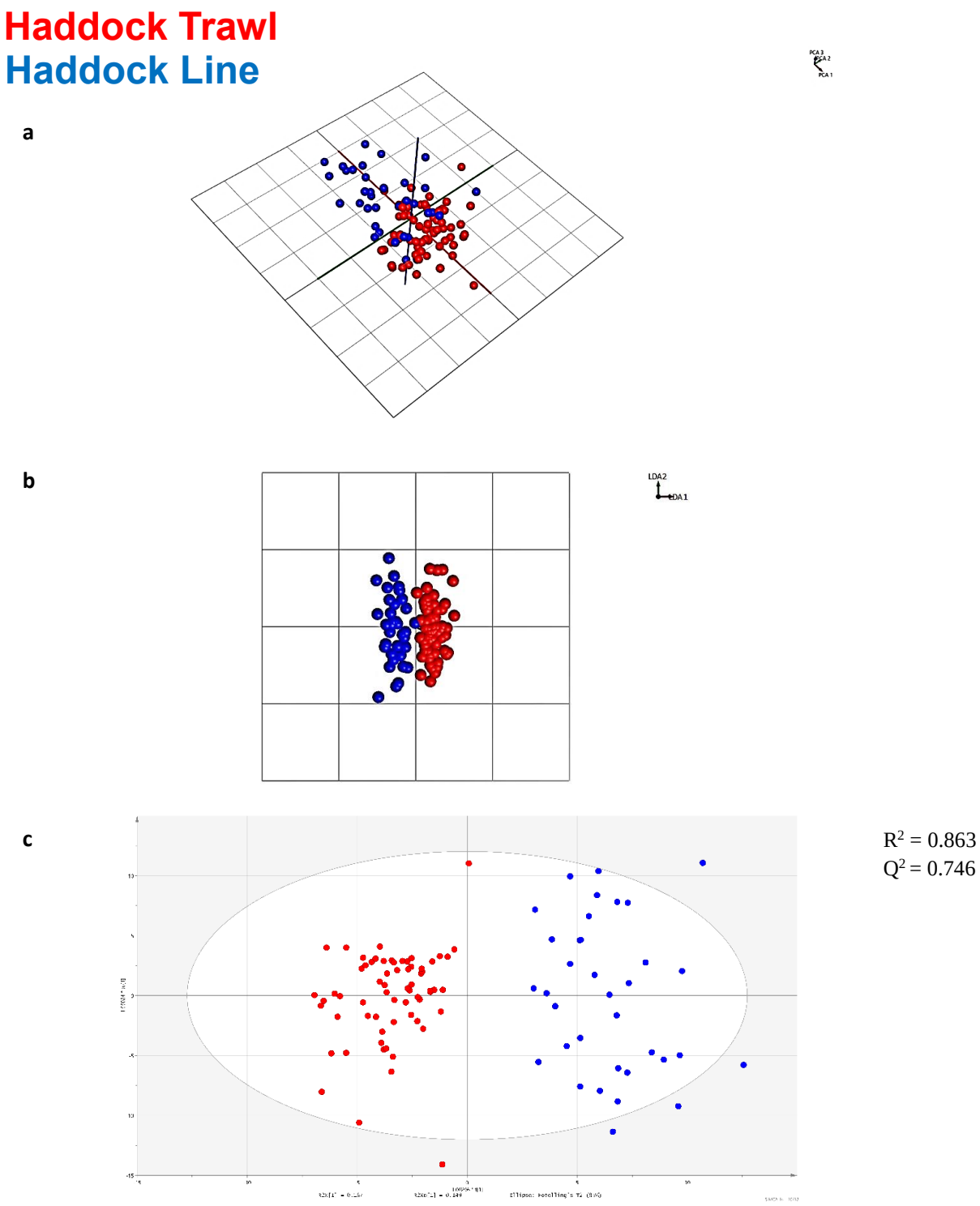


Figure 3.3. (a) Principal component analysis (PCA) (20 PCA components), (b) linear discriminant analysis (LDA) (2 LDA components) and (c) orthogonal partial least squares-discriminant analysis (OPLS-DA) (1 latent and 3 orthogonal components) models generated using the prototype software and SIMCA 14. All models were generated using a bin of 0.5Da and the mass range m/z 600-950 of the fish samples with clear separation of the two catch methods; haddock trawl (red) and haddock line (blue) evident within the 2-D LDA and 3-D OPLS-DA models.

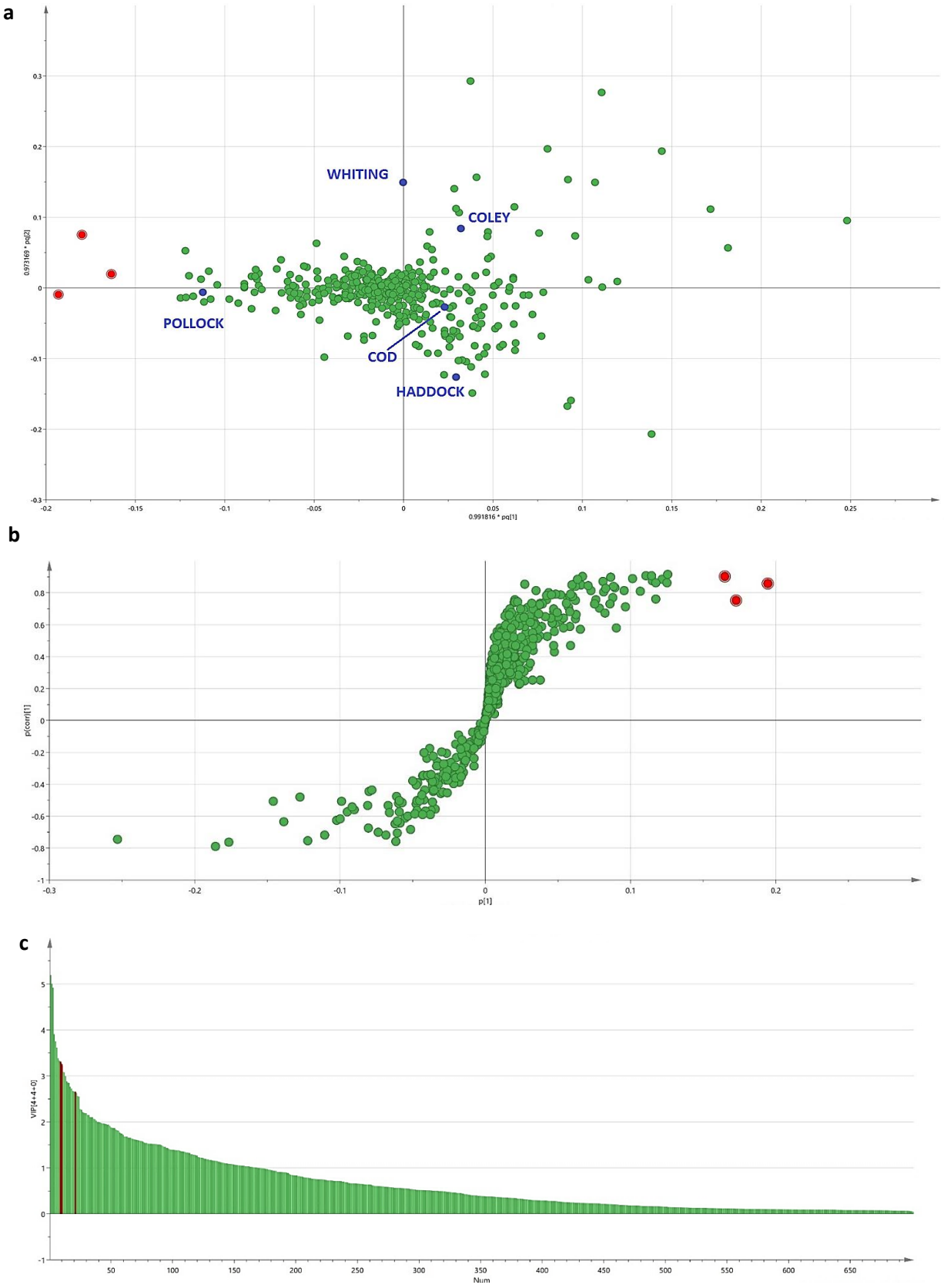


Figure 3.4. Method to identify ions which are found predominately within pollock compared to that of the other four species of fish; (a) a PCA bi-plot identifying the average position of each species of fish

(blue markers) in a PCA score plot and the relevant ions (green markers) that contribute most to their positioning (PCA loading plot); (b) a S-plot of pollock v the other species of fish identifying the ions that are found predominately in pollock; (c) a VIP graph of all 701 ions analysed in the multivariate dataset. The three ions identified (red) have great significance (VIP > 1, S-plot |p| > 0.03 and S-plot |p(corr)| > 0.5) towards the dataset and explain the separation of pollock from the other four spieces of fish within the PCA score plot. Based on MS/MS fragmentation, two of the three ions (m/z 629.5 and 667.5) could not be assigned a putative identification. However, m/z 655.5 $[2M-H]^-$ was identified as a dimer of docosahexaenoic acid (DHA) (m/z 327.21 $[M-H]^-$). Table 3.2 identifies all fragment ions.

Species	m/z (Da)	Collision energy (V)	Ion	Lipid class	Fragment(s) (m/z – Da)	Putative identification
Cod	788.5	30	$[M-H]^-$	PE	327.24	22:6/18:1
					281.25	
					153.00	
				PS	283.26	18:1/18:0
					281.25	
					153.00	
				PS	309.28	20:1/16:0
					255.23	
					153.00	
Coley	817.5	35	N/A	N/A	327.24	N/A
					283.25	
					281.25	
					255.23	
					229.20	
Haddock	810.5	35	$[M-H]^-$	PE	327.24	22:6/20:4
					303.24	
					283.25	
					153.00	
				PE	301.22	22:5/20:5
					257.23	
					153.00	
				PS	303.24	20:4/18:0
					283.25	
					153.00	
Pollock	629.5	20	N/A	N/A	327.24	N/A
					301.22	
					283.25	
	655.5	15	$[2M-H]^-$	FA	327.24	22:6
					283.25	
					229.20	
	667.5	25	N/A	N/A	339.21	N/A
					327.24	
					301.22	
					283.25	
					257.24	
Whiting	790.5	30	$[M-H]^-$	PE	327.24	22:6/18:0
					283.25	
				PS	283.25	18:0/18:0
			N/A	N/A	701.42	N/A
					480.33	
					463.24	
					255.25	

Table 3.2. Putative identifications of the three pollock ions identified in figure 3.4 and the ion found to be most significant for the separation of the other four species of fish in the chemometric models. Two different classes of phospholipids; phosphatidylethanolamine (PE) and phosphatidylserine (PS) were found to be the most likely identification for the ions with the only exception being the pollock ion m/z 655.5 which is believed to be a dimer of docosahexaenoic acid (DHA) (m/z 327.21 $[M-H]^-$). All ions had VIP values > 1, S-plot |p| values > 0.03 and S-plot |p(corr)| values > 0.5 thus justifying selection.

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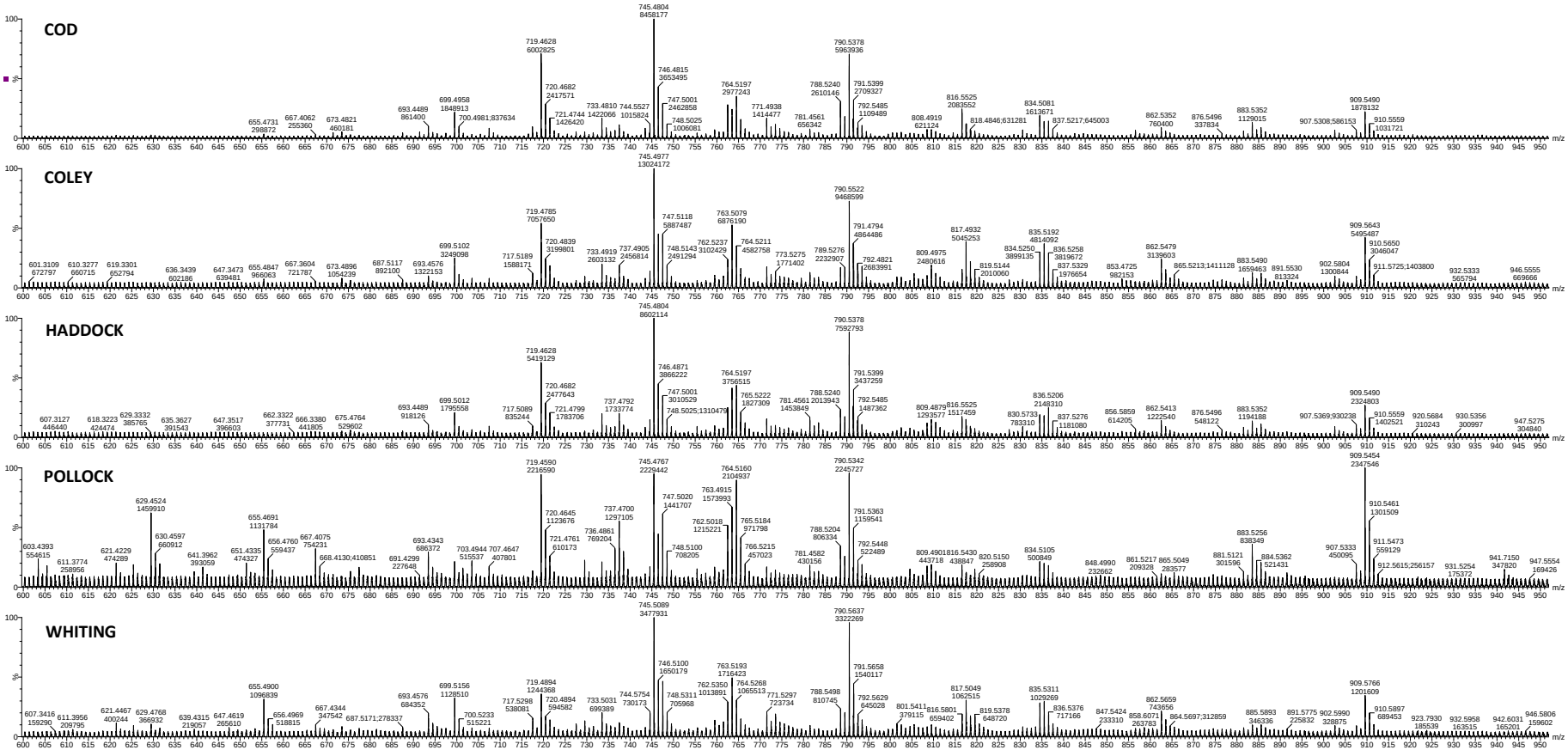
Chapter 3

3.8 Appendix



S1 - Raw background subtracted data (m/z 600-950) obtained from cod, coley, haddock, pollock and whiting fish samples acquired using REIMS operated in negative ion and sensitivity mode.

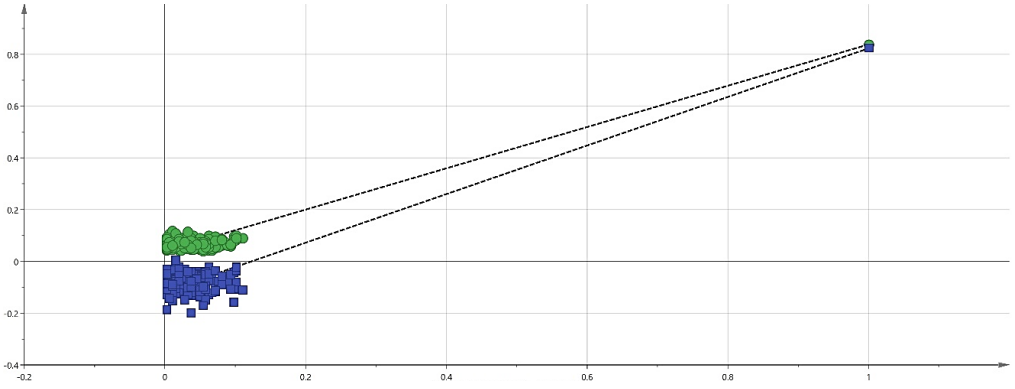
	Cod	Coley	Haddock	Pollock	Whiting	Outlier	Total	Correct classification rate (%)
Cod	193	0	0	0	0	1	194	99.48
Coley	0	51	0	0	0	0	51	100.00
Haddock	0	0	132	0	0	1	133	99.25
Pollock	0	0	0	50	0	0	50	100.00
Whiting	0	1	0	0	49	0	50	98.00
Total							478	99.37

S2 - Results from the leave-20%-out cross-validation of the PCA-LDA speciation models generated using the prototype software in which an overall correct classification rate of 99.37% was achieved. Of the 478 samples analysed, only 3 were not assigned the correct species classification.

	Cod	Coley	Haddock	Pollock	Whiting	Outlier	Total	Correct classification rate (%)
Cod	192	1	0	0	1	0	194	98.97
Coley	0	51	0	0	0	0	51	100.00
Haddock	0	0	133	0	0	0	133	100.00
Pollock	0	0	0	50	0	0	50	100.00
Whiting	0	1	0	0	49	0	50	98.00
Total							478	99.37

S3 - Results from the misidentification table of the speciation OPLS-DA model generated using SIMCA 14 in which an overall correct classification rate of 99.37% was achieved. Of the 478 samples analysed, only 3 were not assigned the correct species classification.

a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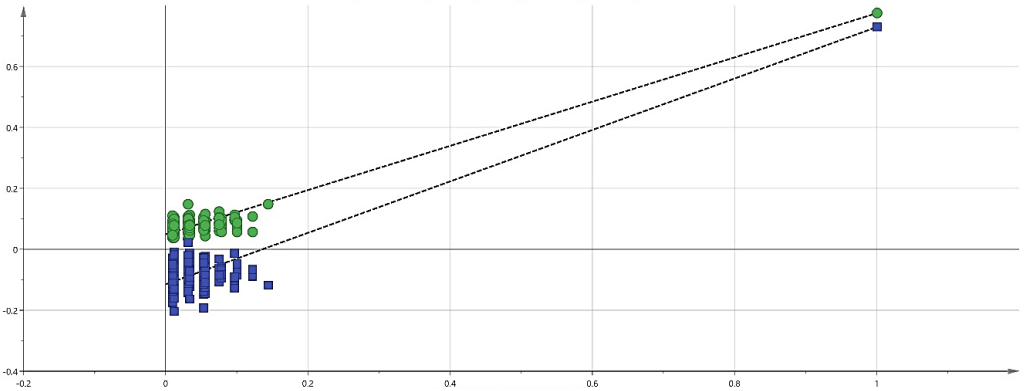
b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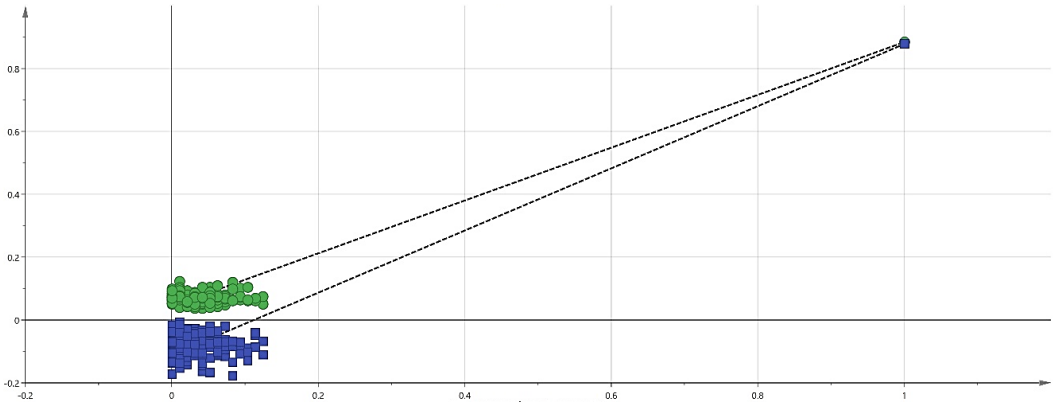
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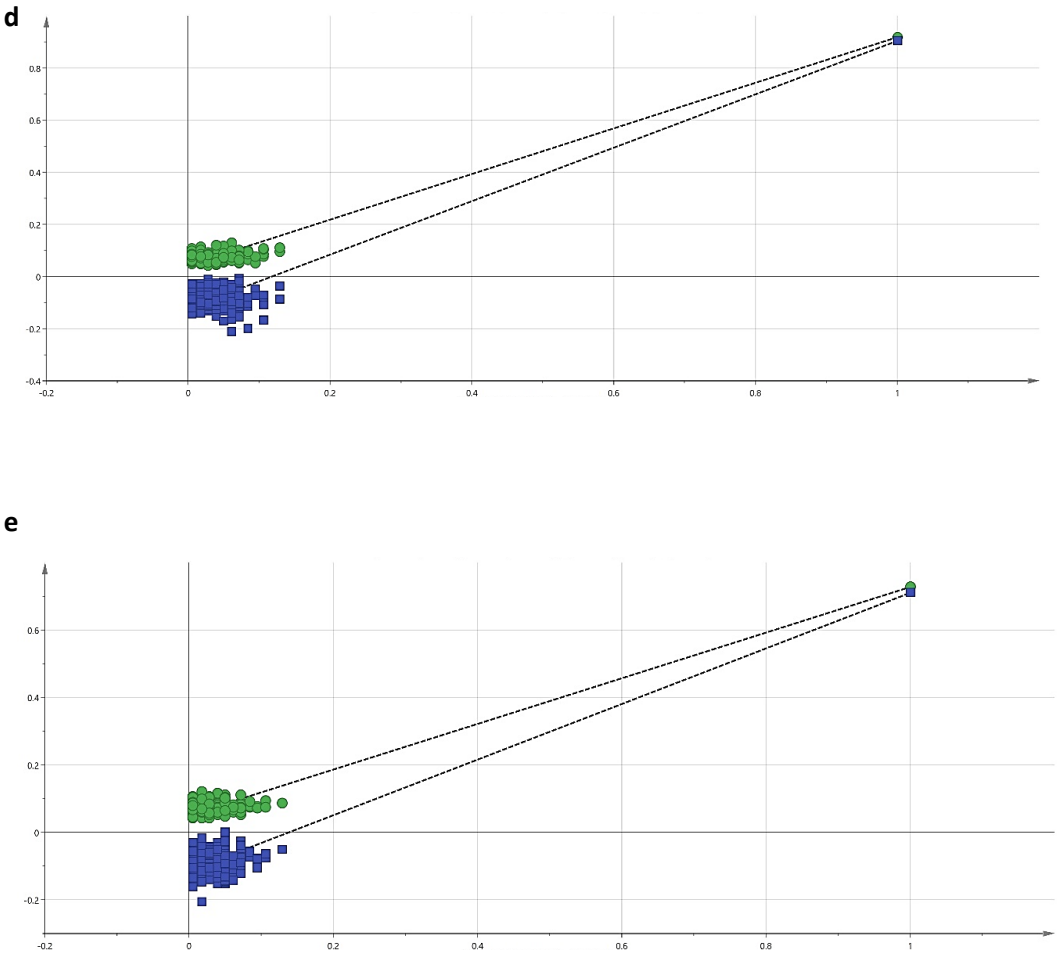


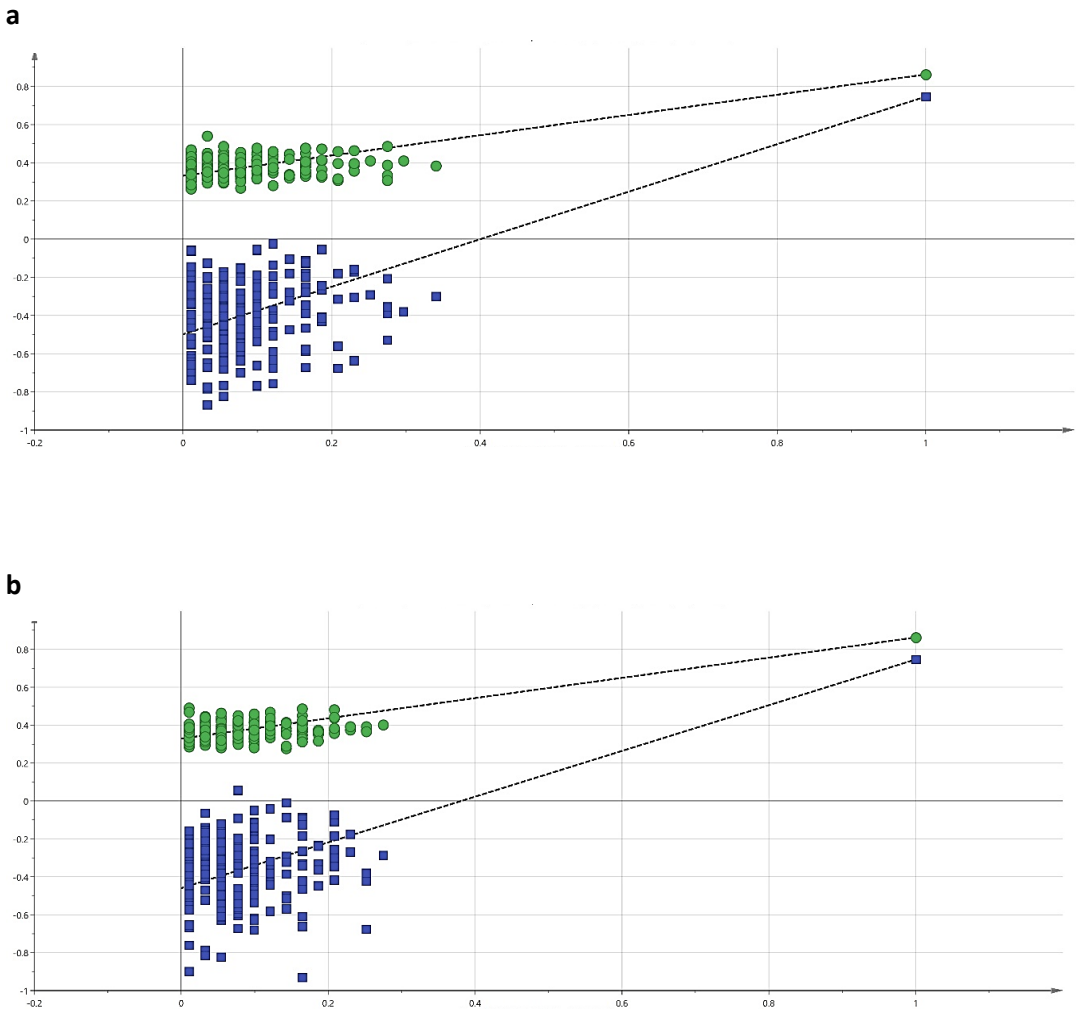
S4- Permutation tests for each species of fish within the OPLS-DA model (figure 3.1c); (a) cod; (b) coley; (c) haddock; (d) pollock and (e) whiting. All tests were carried out using 200 permutations with each Q^2 regression line (blue) intercepting the y-axis beneath the origin suggesting that the OPLS-DA model was not over-fitted.

	Cod	Coley	Haddock	Pollock	Whiting	Outlier	Total	Correct classification rate (%)
Cod	21	0	0	0	0	1	22	95.45
Coley	0	20	0	0	0	0	20	100.00
Haddock	0	0	20	0	0	0	20	100.00
Pollock	0	0	0	20	0	0	20	100.00
Whiting	0	0	0	0	17	0	17	100.00
Total							99	98.99

S5 - Statistical validation of the speciation models using the prototype software cross validation ensuring the results from the prototype recognition software were accurate. All samples were assigned the correct fish species except one cod sample which was identified as an outlier resulting in an overall correct classification rate of 98.99%.

	Haddock Line	Haddock Trawl	Total	Correct classification rate (%)
Haddock Line	33	2	35	94.29
Haddock Trawl	3	62	65	95.38
Total			100	95.00

S6 - Results from the misidentification table of the catch method PCA-LDA model generated using the prototype OMB software in which a 95% correct classification was obtained.

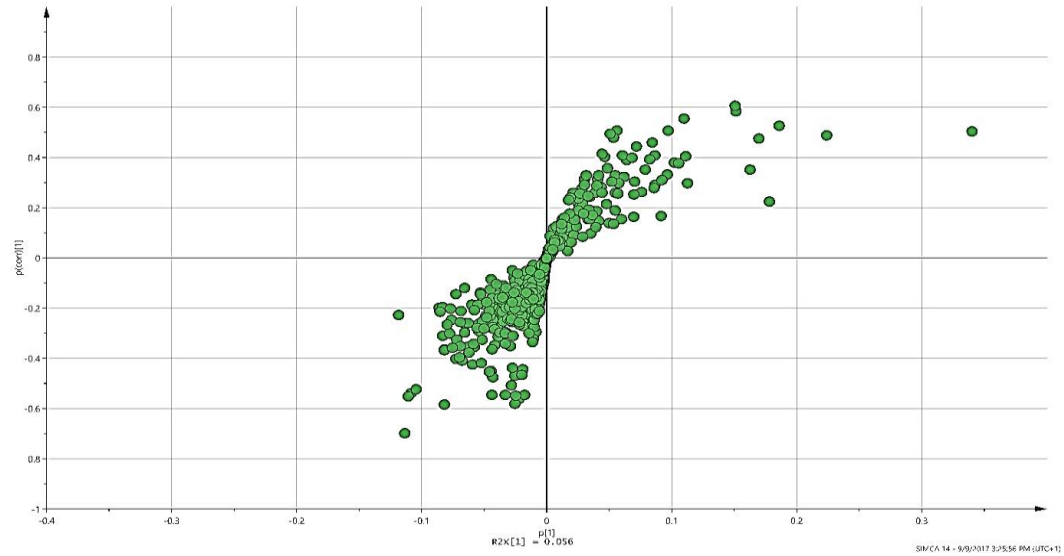


S7 - Permutation tests for each catch method of haddock within the OPLS-DA model (figure 3.3c); (a) haddock line and (b) haddock trawl. All tests were carried out using 200 permutations with each Q^2 regression line (blue) intercepting the y-axis beneath the origin suggesting that the OPLS-DA model was not over-fitted.

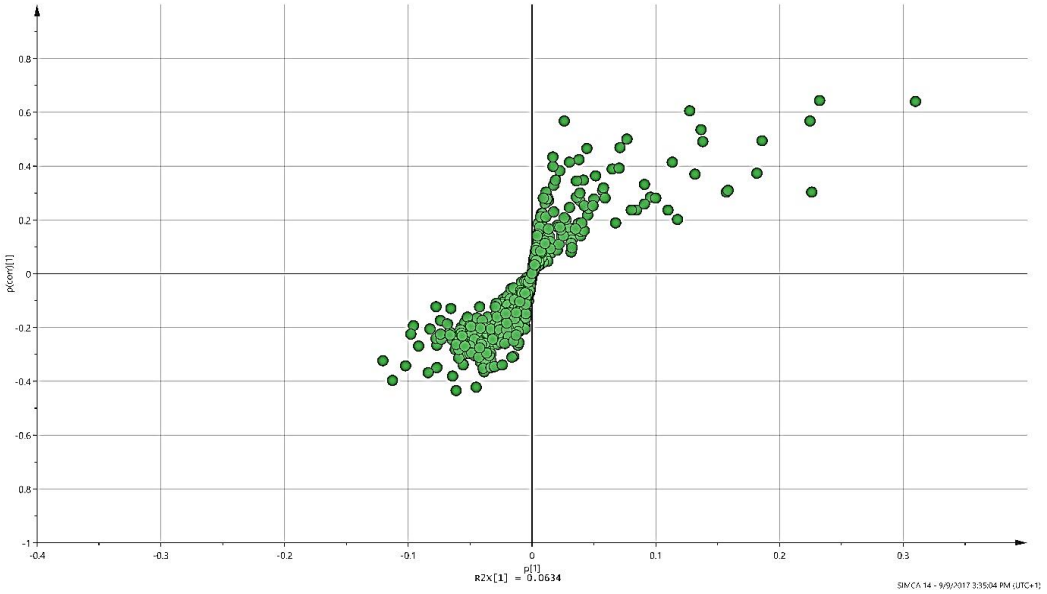
OPLS-DA model	Latent component	Orthogonal component	R^2 (cum)	Q^2 (cum)	RMSECV
Cod v other species	1	5	0.903	0.886	0.166
Coley v other species	1	9	0.909	0.857	0.117
Haddock v other species	1	4	0.930	0.923	0.124
Pollock v other species	1	5	0.954	0.932	0.080
Whiting v other species	1	6	0.874	0.829	0.127

S8- Values of the statistical parameters obtained for the different OPLS-DA models of each species of fish against the other four species under investigation generated using REIMS in negative ionisation mode to identify ions of significance.

a



b



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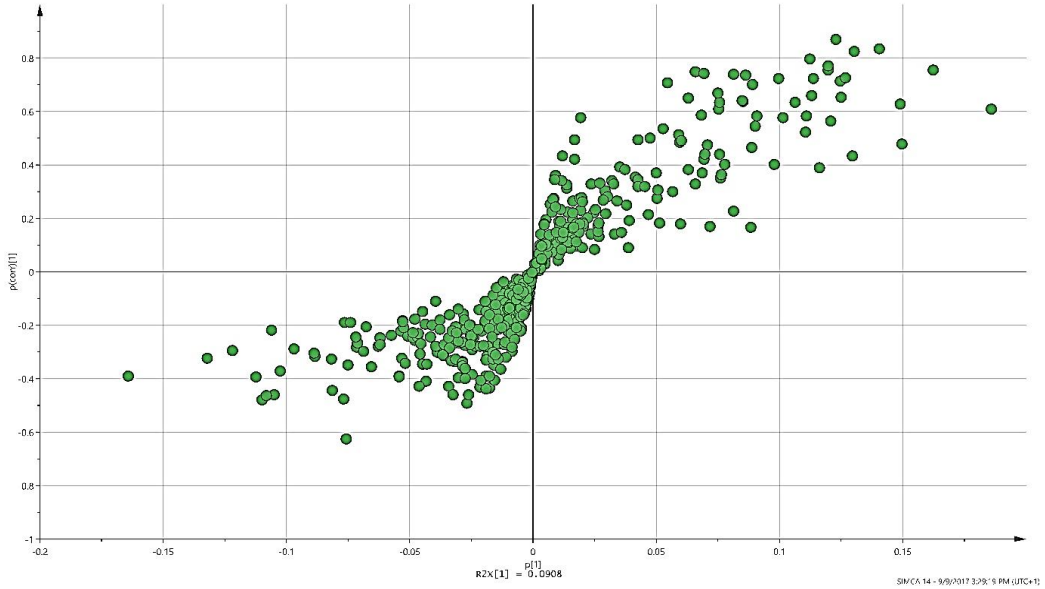
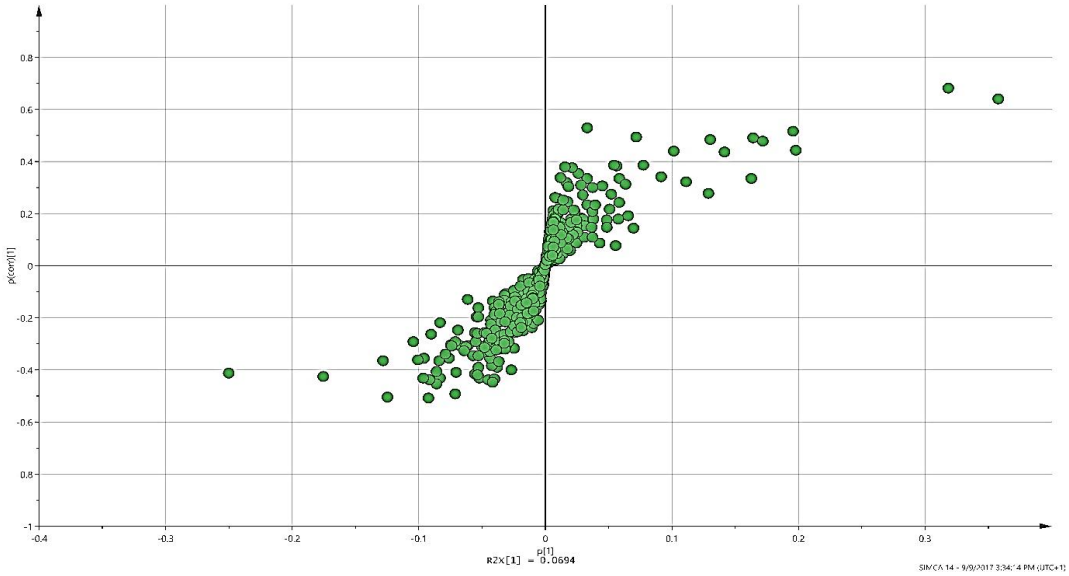


d



S9 – S-plots of (a) cod; (b) coley; (c) haddock and (d) whiting versus the other four species of fish under investigation identifying ions of significance which contribute to the separation of the five species of fish within the various chemometric models. Similar to that of the relevant ions identified within the pollock s-plot, all ions labelled in red are of great significance and contribute greatly to the separation of the five fish classes within the various chemometric models. Additionally, all ions have a VIP >1.

4. Real time identification of the adulteration of processed meat using rapid evaporative ionisation mass spectrometry

4.1 Abstract

Adulteration of processed meat products with low cost materials is a huge issue globally. Thus, product substitution can be in full or partially but must be conducted at levels of at least 10-20% for criminals to make a profit from the fraud. A range of analytical platforms are capable of such detection limits though long and often complex sample preparation and assay running times are required. Spectroscopic techniques circumvent these issues to some degree however, their inability to identify species-specific markers is a major short coming. In this study, we present an effective, near real-time method to identify minced beef adulteration with goat, lamb and pork using rapid evaporative ionisation mass spectrometry (REIMS). Detection of meat adulteration/substitution at levels ranging from 2-20% were identified depending on how the samples were prepared. The identification of multiple meat species within a sample was also correctly identified at levels of adulteration ranging from 25% to 33%. Database search associated with MS/MS fragmentation did not result in the identification of any unique species-specific markers. However, many ions were found to occur more prominently in certain species and therefore, assigned lipid classes. They were identified as phosphatidic acid (PA) phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) species. Thus, we present a technique using a rapid fingerprinting approach that could serve to detect meat adulteration in near real time.

4.2 Introduction

Rapid evaporative ionisation mass spectrometry (REIMS) enables direct ionisation of samples using an electrosurgical knife, bipolar forceps or infrared (IR) laser under ambient conditions. Thus, it is categorised as an ambient mass spectrometry (AMS) technique, a field of analytical chemistry first identified in 1998 through the conceptualisation of paper spray-mass spectrometry (PS-MS),[1] but not demonstrated until 2004 by desorption electrospray ionisation-mass spectrometry (DESI-MS).[2] Whilst most AMS techniques require some minimalistic form of sample preparation, REIMS does not. Combined with near-instantaneous mass spectrometric analysis, this innovative technology circumvents many intrinsic constraints encountered by others. Initially conceptualised for in vivo, in situ analysis of human tissues,[3] REIMS has found applications in cancer analysis,[4] bacterial and microorganism identification including that of *Escherichia coli* (E. coli).[5,6] Most recently it has been successfully applied to food analysis including the adulteration of beef with horse and venison, and the detection of boar taint, a contemporary off-odour found in the meat of uncastrated male pigs.[7,8] This rapid fingerprinting technique profiles fatty acids and phospholipids and uses them for the identification or separation of a class.

Meat is the most vulnerable commodity to food fraud within the European Union (EU),[9] as the 2013 European horsemeat scandal demonstrated. The scale of fraud was substantial and led to a significant increase in global media coverage as well as widespread decline in consumer confidence. The seriousness of the scandal within the United Kingdom (UK) led to an independent review of Britain's food system, the 'Elliott review', which addressed weaknesses within the UK food supply network that had allowed such fraud to occur and how these could be improved to prevent further such incidences.[10] Like most food commodities, meat adulteration can take several forms and are often categorised into four main areas; (1) meat origin which includes sex, cut, breed and age; (2) meat substitution; (3) meat processing or treatment and (4) non-meat ingredient additions.[11] Non-processed meat products are mostly prone to fraud by means of geographic origin, sex and breed, whilst processed meat products

such as burgers, meatballs and readymade meals are most susceptible to species adulteration and offal meat (heart, kidney and lung) additions.[12,13] That is because it is easy to pass of the presence of a foreign object within a minced or blended sample. Other food commodities such as herbs and spices have recently been found to suffer a similar fate.[14,15]

Detection of meat adulteration has been studied extensively using a wide range of analytical techniques and profiling approaches. Genomic profiling using polymerase chain reaction (PCR) is the one of the most commonly used techniques as it is a DNA-based technology.[16] DNA is thought to be highly stable thus making it an appropriate molecule for species detection and identification within meat products. The stability of DNA through various freeze/thawing cycles is significant in comparison to metabolomic studies in which metabolites are thought to vary drastically. Although PCR produces excellent sensitivity in unprocessed foods, the conditions that meat products endure through various processing methods (temperature and pH changes) can lead to degradation of DNA. With most meat adulteration cases occurring in processed products, this is a severe limitation. Alongside PCR, ELISA is another popular technique used for such studies. Capable of analysing proteins, peptides, antibodies and hormones, it has been demonstrated that the presence of one meat in another meat at levels <1% are detectable in raw, cooked and autoclaved meat products.[17] However, a limitation to immunoassays is the need for specific antibodies as the specificity in highly processed samples can be critical thus resulting in false positive and negative results.[18] Liquid chromatography-mass spectrometry based proteomic profiling studies are also commonly undertaken with electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI) being the most common ionisation methods.[19,20] ESI is especially popular due to its ability to create multiply charged species which enables analysis of high molecular weight proteins. Additionally, with ESI being a ‘soft’ ionisation technique it involves minimal fragmentation allowing the analysis of intact proteins. Whereas DNA is suspect in processed products, proteins are relatively more stable. This allows species specific proteins or peptide proteins to be identified and used as markers. However, the potential coelution of proteins is an issue that can occur thus leading to ion suppression. Other analytical

techniques such as stable isotope ratio analysis and spectroscopic techniques such as near infrared (NIR) and Fourier transform infrared (FT-IR) have also been utilised for similar studies.[21-23]

In comparison to genomic and proteomic profiling, there are a shortage of lipidomic based studies aimed at detecting meat adulteration. Although lipidomics is a branch of the metabolome, such is the complexity of lipid classes and their interactions that it possible to separate out meat species based on their lipid profile. The purpose of this study was to identify whether REIMS, a technique whose spectra are dominated by intact lipids, in conjunction with chemometric analysis could differentiate between four different meat species (beef, goat, lamb and pork) and establish if there were unique ions that were responsible for such separation. Finally, we wanted to establish what limits of detection (LOD) were achievable by analysing raw beef burgers which were adulterated with the other three meat species at various adulteration levels and compare our findings to those previously reported using other AMS and conventionally used techniques.

4.3 Methods

4.3.1 Samples

All tissue samples of beef (n=62), goat (n=22), lamb (n=30) and pork (n=20) were sourced from trusted suppliers, stored at -80^{o}C and minced using a blender prior to making pure and adulterated burgers.

4.3.2 Assembly of adulterated beef burgers

Minced adulterated beef ‘burgers’ (20g) were prepared starting at 50% adulteration (40g) using one beef and three adulterant samples and then serial diluting down to obtain levels of 20, 10, 5, 2, 1 and 0.1% adulteration using a blender to homogenise the samples. Burgers containing multiple species were also prepared at levels of 33% and 25%. Three ‘burgers’ were made for each adulteration level which all contained different beef and adulterant samples. Additional adulterated beef ‘burgers’ (20g) at levels of 20, 10, 5, 2, 1 and 0.1% adulteration were individually made using a blender to homogenise the samples. Prior to

REIMS analysis all burgers were thawed at room temperature for two hours in the fumehood where the REIMS cutting took place.

4.3.3 Instrumentation

A Waters REIMS source (Waters Corporation, Wilmslow, UK) was coupled to a Xevo G2-XS quadrupole time-of-flight (QTof) mass spectrometer (Waters Corporation, Wilmslow, UK) which was operated in negative ion and sensitivity mode. Mass spectra data were acquired over the range m/z 200-1200 with a scan time of 0.5s. The REIMS source was connected to a monopolar electrosurgical knife (Model PS01-63H, Hangzhou medstar technology Co, Ltd, Jiaxing City, China) through a 3m long, 1cm. diameter ultra-flexible tubing (evacuation/vent line). Electrosurgical dissection in all experiments were performed using an Erbe VIO 50 C generator (Erbe Medical UK Ltd, Leeds, UK). The generator was operated in 'autocut' mode with a power setting of 30W. All samples were cut on the return electrode and a venturi gas jet pump driven by nitrogen (1 bar) evacuated the aerosol produced at the sample site towards a heated kanthal coil that was operated at 40V. A lockmass solution of Leucine Enkephalin (LeuEnk) (m/z 554.2615) (0.1ng / µL) in isopropanol (IPA) was infused using a Waters Acquity UPLC I-class system (Waters Corporation., Milford, MA, USA) at a continuous flow rate of 0.2 mL/min for accurate mass correction. Prior to analysis the mass spectrometer was calibrated using 0.5mM sodium formate solution (90% IPA) at a flow rate of 25 µL/min for two minutes. Dependent on the size, each tissue sample was cut 10-15 times for reproducibility with each cut lasting approximately 3-5s. This enabled multiple locations on each tissue sample to be analysed. The delay between sampling and appearance of a signal was ≈2s, with no carry-over effects visible between each burn and/or sample.

4.3.4 Data pre-processing and analysis.

Principal component analysis (PCA), an unsupervised technique, linear discriminant analysis (LDA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), both supervised techniques, were used to build the qualitative speciation models.

Raw data generated by the mass spectrometer were pre-processed using a prototype model builder recognition software, OMB version 1.1.1017.0 (Waters Research Centre, Budapest, Hungary) that used standard Masslynx pre-processing algorithms (Waters). Data were lockmass corrected using LeuEnk (m/z 554.2615) and normalised (Total Ion Count - TIC) before being exposed to multivariate analysis. All chemometric models were calculated using the mass region of m/z 600-950, a spectral intensity threshold of $2e^6$ counts and a bin width of 0.05 Da. PCA was used to reduce the dimensionality of the data prior to LDA analysis using the first 25 PCA components. The OMB software enabled a leave-20%-out cross-validation of the PCA-LDA score plots using 80% of the samples. The remaining 20% were assigned a classification using a standard deviation of 20σ. The process was repeated five times enabling each sample to be left out once from the model building process. If a sample was outside the standard deviation range of 20σ for all classes, then they were marked as outliers.

Alongside OMB analysis, raw mass spectrometric data were pre-processed, lockmass corrected and background subtracted using the Progenesis bridge conversion tool (Waters, Wilmslow, UK) before being imported to Progenesis QI 2.0 software (Waters, Wilmslow, UK). A filter of 2.5 was applied to supress background noise, whilst preserving the significant peaks in the data. Sensitivity was set at automatic with peak width set to 0.08.

The processed matrices generated within the OMB and Progenesis software's were exported to SIMCA 14 (Umetrics, Umea, Sweden) allowing data to be exposed to further chemometric functions such as OPLS-DA. All data were mean-centred, pareto scaled and grouped accordingly into the four-meat species. R^2 (cumulative), and Q^2 (cumulative) were used to determine the validity of the models. R^2 (cum) indicates the variation described by all components in the model and Q^2 (cum) is a measure of how accurately the model can predict class membership. Permutation tests were carried out to ensure the models were not over-fitted. Individual OPLS-DA models of each species of meat against the other three species enabled S-plots to be created which demonstrate the importance of each variable (ions) to each observation (species/class). S-plot |p| and |p(corr)| values were used to validate ion selection for MS/MS analysis with the contribution of each variable (ion) to the variance of the

observations being determined by the |p| value and the reliability of each variable for group separation identified by the |p(corr)| value.

4.3.5 Real time recognition of samples

The PCA-LDA models created using the prototype OMB software were exported to the OMB prototype recognition software version 1.1.1017.0 (Waters Research Centre, Budapest, Hungary) allowing for real-time identification of samples. Raw data files were acquired and ran live though the software providing a near-instantaneous identification, excluding the delay between sampling and appearance of a signal which was ≈2s. A standard deviation of 20σ was used for class assignment. The spectral intensity limit was set at $6e^7$ counts thus ensuring that only the cuts were assigned a species classification and not any background noise.

4.4 Results

4.4.1 Untargeted analysis of beef, goat, lamb and pork burgers

Raw spectrometric data (figure 4.1) obtained from beef (n=50), goat (n=10), lamb (n=18) and pork (n=8) samples were subjected to multivariate analysis using a prototype OMB model building software in which principal component analysis (PCA) and linear discriminant analysis (LDA) (figure 4.2) models were generated. 13 PCA and 3 LDA components were used and all models were generated using mass range m/z 600-950 and bin of 0.05 Da. A leave-20%-cross validation of the dataset resulted in a 100% cross validation (supplementary information S1). The data matrix was exported to SIMCA 14 enabling orthogonal partial least squares-discriminant analysis (OPLS-DA) models to be generated in which 3 latent and 3 orthogonal components were used (supplementary information S2). R^2 and Q^2 values of 0.924 and 0.898 were obtained respectively indicating both good quality of fit and predictivity towards new data. Permutation tests also demonstrate the robustness of the models (supplementary information S3).

4.4.2 Identification of species markers

Filtering of the raw spectrometric data using Progenesis QI 2.0 reduced ambient background noise whilst preserving the most significant peaks in the data. Peak picking followed before

exporting the data matrix to SIMCA 14 allowing individual OPLS-DA models of each species of meat against the other three species to be generated (supplementary information S4). No unique ions were identified but several ions were selected which were thought to be responsible for species separation within the chemometric models. The selected ions were cross-referenced to LipidBlast and Metlin with a tolerance set at 5ppm to identify the lipid class of each ion. MS/MS fragmentation was carried out on the selected ions to assign putative identifications within each lipid class. The results are shown in table 4.1.

4.4.3 Detection of individually made adulterated beef burgers

The PCA-LDA models generated using the OMB software were exported to a prototype recognition software allowing for near-instantaneous identification of each burger burn. Beef burgers adulterated with goat, lamb and pork at levels of 20, 10, 5, 2, 1 and 0.1% were individually made using multiple adulterant samples for each burger and homogenised using a blender. One replicate was analysed for each adulteration level. Goat was correctly identified at 2%, pork at 5% and lamb at 10% adulteration. Additionally, burgers containing all four-meat species (25% each) and three meat species (33% each) were made. All species were correctly identified in the three 25% burgers (figure 4.3) and only one false positive was identified in the nine 33% burgers as shown in table 4.2. All samples used to make the adulterated beef burgers, including the bovine samples, were not used in the PCA-LDA model building process.

4.4.4 Detection of adulterated beef burgers made through serial dilution

To mimic the act of food fraud in a real-world environment, burgers were made through a serial dilution process using a blender to homogenise the samples after each dilution. Like the handmade burgers, multiple adulterant samples were utilised for each burger. Raw spectrometric data acquired from beef burgers adulterated with goat, lamb and pork at levels of 50, 20, 10, 5, 2, 1 and 0.1% were simultaneously run live through the software allowing species identification to be identified within seconds of cutting. All samples used to make the adulterated beef burgers, including the beef samples, were not used in the original model building process. Levels of 10% adulteration were detectable in beef burgers substituted with

pork and goat whilst only 20% adulteration was achievable in the beef burgers adulterated with lamb. Three burgers (replicates) were analysed for each adulteration level with table 4.2 providing an overview of the results including those of the 25% and 33% adulterated burgers. Figure 4.4 shows an LDA score plot of all the pure meat and 50% adulterated burger burns demonstrating clear signs of adulteration.

4.5 Discussion

Commercial meat species substitution within processed or minced products is an operation undertaken at levels ranging from ~20% up to 100%to enable criminals to earn substantial additional profits. Although there are concerns with regards to the quantitative abilities of many AMS techniques,[25] this study demonstrates that REIMS is proficient at not only detecting adulteration at levels as low as 2%, but also that species identification can be obtained near-instantaneously (≈2s) without the need for any form of sample preparation (figure 4.3). It is evident that the process of serial dilution impacts the quantitative abilities of the REIMS technology with LOD's of each adulterant being higher compared to those not made through serial dilution. With each adulterant sample being minced/blended three times prior to carrying out the 10% adulteration dilution, such strenuous mixing or blending may have resulted in them being 'pulverised' making it more difficult to detect them. Goat is a much gamier and tougher meat compared to that of lamb and pork and therefore, it is conceivable that it could 'survive' an extra blending. That would explain why all three burgers at 10% adulteration were identified. Perhaps this study has demonstrated that low LOD's (2-5%) are achievable using REIMS but when the products have been heavily processed or mixed vigorously then those levels are higher.

Detection of 10-20% adulteration is sufficient for most reported cases of meat substitution yet lower levels (<1%) are achievable using many conventional techniques such as PCR, ELISA and LC-MS.[16,17,19,20] However, identification of multiple meat species or non-meat samples at extremely low levels (0.1-1%) in supposedly pure commercial meat products is a result of accidental cross contamination and not the deliberate act of food fraud. Therefore, this study

as well as others carried out using different ambient ionisation techniques such as liquid extraction surface analysis-mass spectrometry (LESA-MS) demonstrate that LOD's of 5-10% are very achievable and that they have a prominent role to play in detecting meat adulteration.[7,26]

Unique species-specific ions could not be identified in this study however, table 4.1 shows which were majorly responsible for class separation within the chemometric models. All ions had S-plot |p| values greater than 0.03, |p(corr)| values greater than 0.5 and variable importance for projection (VIP) values > 1 thus validating their selection.[8,27] Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) were the assigned lipid classes for the selected ions and putative identifications within each class were assigned based upon MS/MS fragmentation (table 4.1). Multiple identifications were assigned for each ion due to the lack of chromatographic separation that is witnessed using REIMS. Similar to a previous phospholipid study using high performance LC-MS (HPLC-MS), m/z 742.54 $[M-H]^-$, or m/z 744.5 $[M+H]^+$ in the case of the HPLC-MS study was found to be a prominent phospholipid species within the pork samples. Previously this was identified as PE (18:1/18:1).[28] However, whilst that is a potential identification within this study (table 4.1), MS/MS fragments of deprotonated linoleic acid (m/z 279.23), stearic acid (m/z 283.26) and the loss of the $C_{18:2}$ acyl chain (m/z 480.31) lead us to believe that the prominent PE species in pork maybe PE (18:2/18:0). Due to the presence of additional fatty acid (FA) fragments, m/z 742.54 can also be identified as two different PC $[M-CH_3-H]^-$ adducts - PC (18:2/18:0) and PC (18:1/16:1). FA fragments of oleic acid (m/z 281.25) enabled assignment of PA (18:2/18:1) for m/z 697.48. This was found to be the most abundant phospholipid species in pork compared to that of m/z 699.50 which was the most abundant phospholipid in the other three meat species (figure 4.1). That ion is most likely to be some form of PA (36:2) species. Both linolenic acid (m/z 277.21) and oleic acid led to the assignment of PA (18:3/18:1) to the lamb ion although the MS/MS fragment of linoleic acid also suggests the presence of PA (18:2/18:2). Arachidonic acid (m/z 303.23) was identified in the goat MS/MS fragments which along with the other fragments in table 4.1 led to the

assignment of PC (20:4/16:0), PE (20:4/18:0) and PS (P-16:0/20:4). The presence of m/z 255.23 in all meat samples (figure 4.1) was thought to be palmitic acid (FA (16:0)), a saturated FA commonly found in meats, cheeses and other dairy products.

Although the focus of this study was on the adulteration of minced beef products, the identification of an ion found to occur more prominently in beef (PI (18:1/18:0)) is significant. Processed lamb products have regularly been subjected to adulteration and beef has often been the chosen adulterant. In 2013 the UK Foods Standards Agency (FSA) identified when analysing 145 lamb takeaway meals that 43 had other meats present with 25 of them containing only beef.[29] Therefore, the identification of the beef ion is significant. Likewise, the identification of two ions found to occur at much more abundant levels in pork than the other three species is significant as it too is often used as an adulterant in lamb samples.[30] Other than horsemeat which has been studied extensively since the 2013 scandal, pork is perhaps the most investigated adulterant in beef and lamb products due to the many ethical issues it poses for religious groups.

4.6 Conclusions

In conclusion, REIMS could provide a paradigm shift across authenticity applications by providing real-time and reliable results without the need for any form of sample preparation. Although there are conventional techniques capable of identifying samples at lower levels than REIMS and other AMS platforms, when put into context with the levels of adulteration that are required to make an additional profit, REIMS has a prominent role to play in tackling food fraud. This is further enforced by the speed at which results are obtained, including sample preparation time, which cannot be matched by any conventionally used analytical technique bar spectroscopic ones which are often employed preciously for their rapid results.[22] Whilst spectrometric and spectroscopic techniques have previously been shown to work as a complementary two-tier system approach,[14] the amount of bioinformation that can be acquired using mass spectrometric platforms is vastly superior with species-specific, or in this scenario species-prominent lipid ions being identifiable. On the other hand, the use of large quadrupole

time-of-flight (QTof) mass spectrometers (MS) does result in substantial cost comparisons and the inability to operate in the 'field' is a severe limitation. But, with advances being made towards miniaturisation and fieldable MS, it is conceivable that REIMS could be coupled to a cheaper and more portable technique soon.[31]

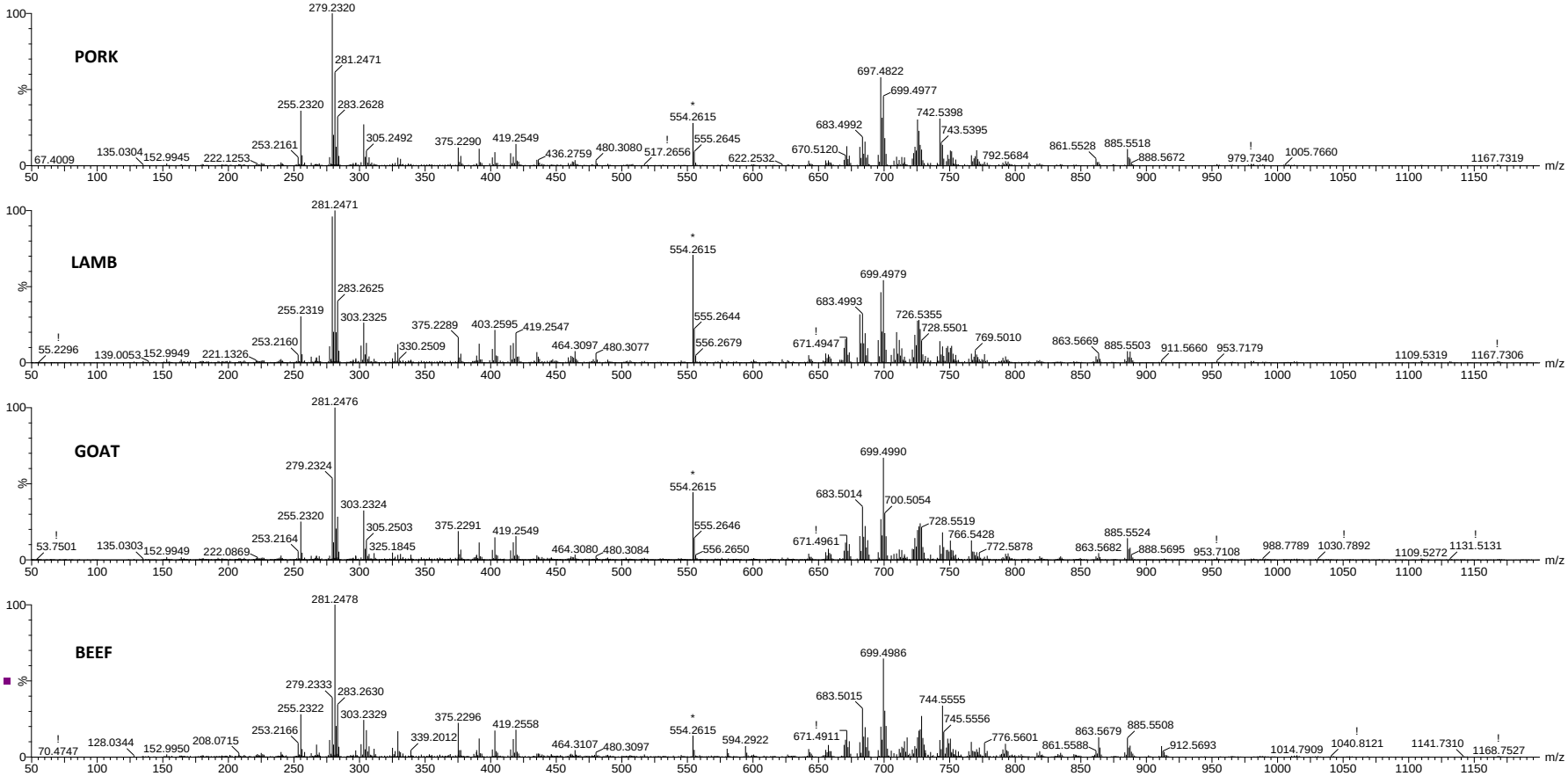


Figure 4.1. Raw background subtracted and lockmassed (m/z 554.2615) data of beef, goat, lamb and pork samples. Fatty acids dominate the lower mass rang (m/z 200-450) whilst phospholipids are situated in the mass range m/z 600-950.

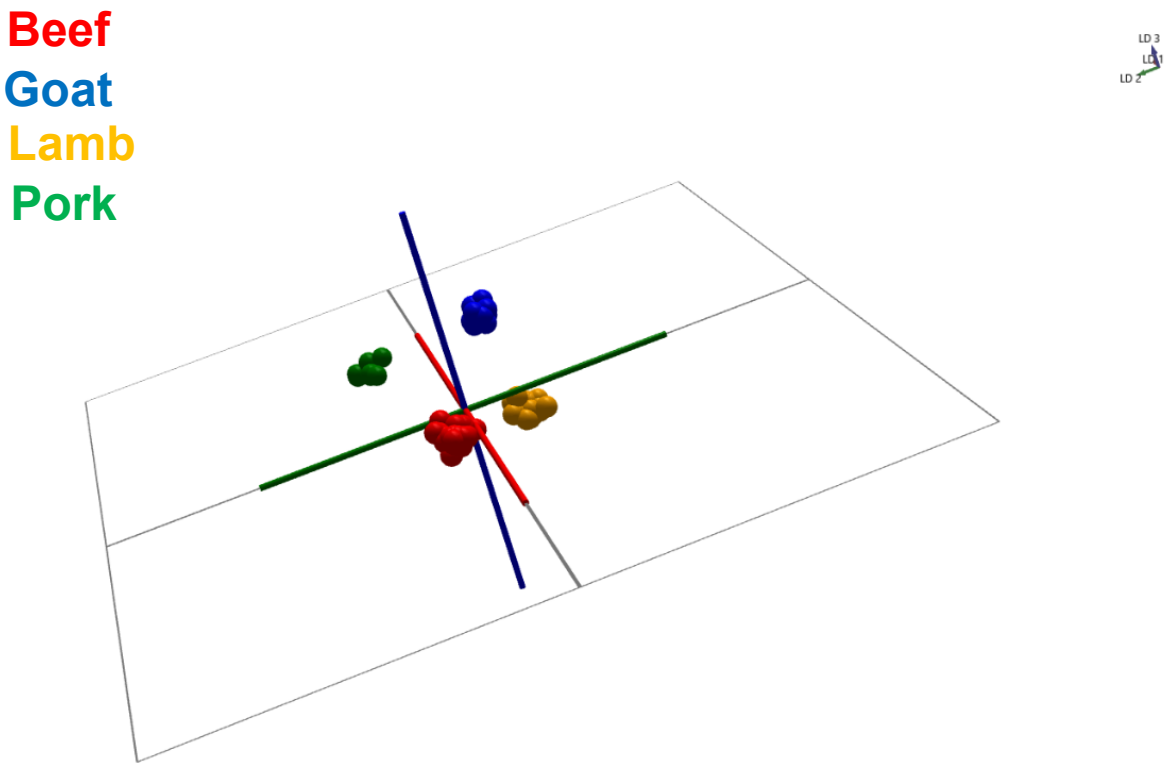


Figure 4.2. Linear discriminant analysis (LDA) score plot (3 LDA components) generated using the prototype OMB model building software using the mass range m/z 600-950 and a bin of 0.05 Da. Clear separation can be seen between the beef (red), goat (blue), lamb (orange) and pork (green) samples in which each data point represents one sample.

Species	m/z (Da)	|p| value	|p(corr)| value	Collision energy (V)	Lipid class	Ion	Fragment(s) (m/z – Da)	Putative identification
Beef	863.57	0.14177	0.70046	40	PI	$[M-H]^-$	599.32 581.31 579.29 283.26 281.25 241.01 223.00	18:1/18:0
Goat	766.54	0.21593	0.57581	30	PE	$[M-H]^-$	480.30 462.29 303.23 283.26 259.24	20:4/18:0
					PS		375.23 303.23 259.24	P-16:0/20:4
					PC	$[M-CH_3-H]^-$	480.30 462.29 303.23 259.24 255.23 224.07	20:4/16:0
Lamb	695.47	0.23517	0.7797	25	PA	$[M-H]^-$	435.25 281.25 277.21	18:3/18:1
					PA		415.23 279.23	18:2/18:2
Pork	697. 48	0.45611	0.88172	30	PA	$[M-H]^-$	435.25 417.24 415.23 281.25 279.23	18:2/18:1
							437.27 419.26 283.27 277.22	18:3/18:0
					PE	$[M-NH_3-H]^-$	279.23 255.23	18:2/16:0
	742.54	0.37881	0.92351		PE	$[M-H]^-$	480.31 462.30 283.26 279.23 140.01	18:2/18:0
					PE	$[M-H]^-$	281.25 140.01	18:1/18:1
					PC	$[M-CH_3-H]^-$	504.31 480.31 462.30 279.23 255.23 224.07 168.04	18:2/16:0
					PC		488.31 281.25 253.22 224.07 168.04	18:1/16:1

Table 4.1. Phospholipid identifications of selected ions found to play a prominent role in the separation of the four-meat species within the PCA and LDA (figure 4.2) score plots. Additionally, the S-plot |p| and |p(corr)| values for each ion are stated validating their selection. Fatty acid $C_{x:y}$ (x = carbon number; y = number of double bonds); $C_{16:0}$ = palmitic acid; $C_{16:1}$ = palmitoleic acid; $C_{18:0}$ = stearic acid; $C_{18:1}$ = oleic acid; $C_{18:2}$ = linoleic acid; $C_{18:3}$ = linolenic acid; $C_{20:4}$ = arachidonic acid.

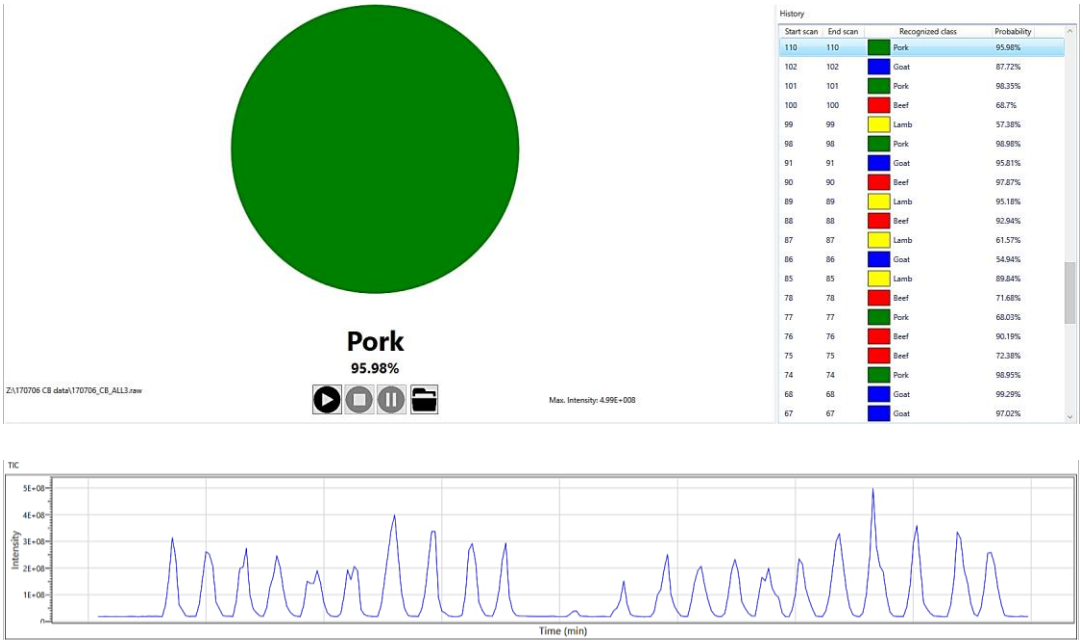


Figure 4.3. Raw data was acquired and simultaneously ran through the prototype recognition software providing identifications of each burger near-instantaneously (≈2s). The burger being analysed in this figure contained all four meat samples at equal percentages of 25%. As can be seen one of the scans selected has been identified as pork. All four species were correctly identified within the sample, as shown on the right-hand side, with each of them receiving roughly the same amount of identifications. The chromatogram beneath shows that 21 burns for taken for that sample.

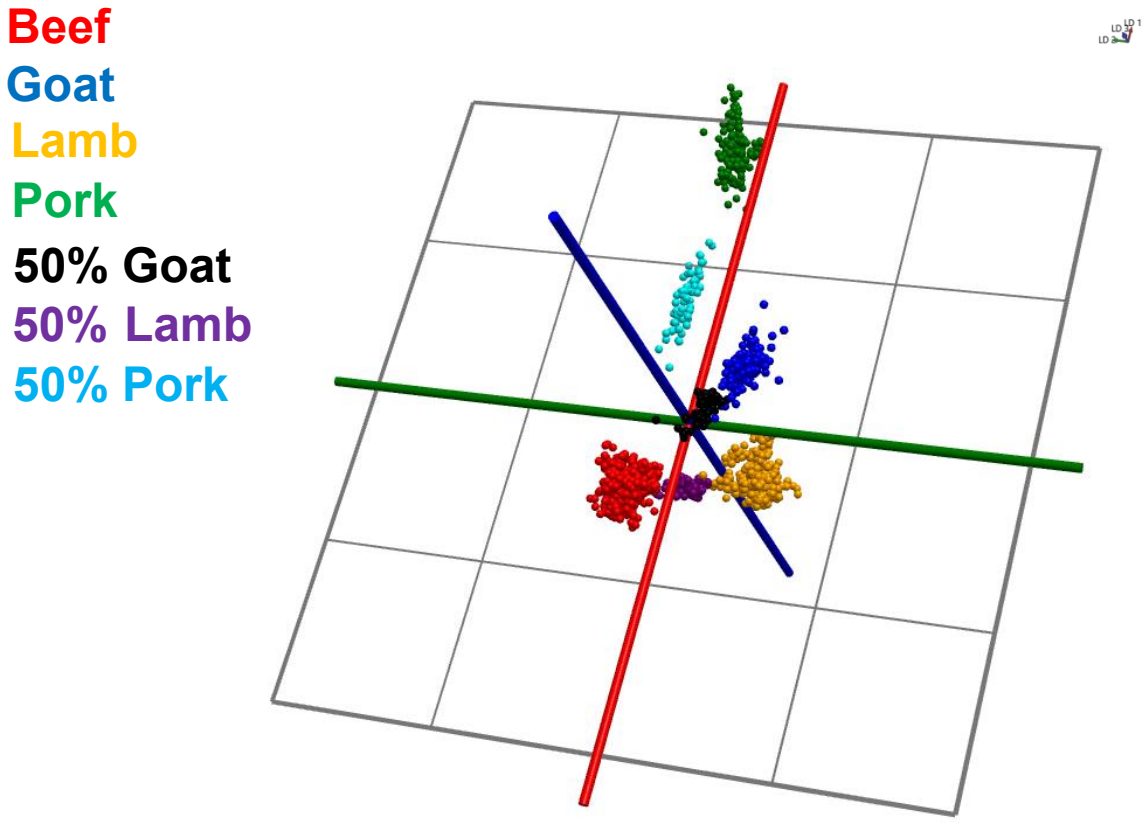


Figure 4.4. Linear discriminant analysis (LDA) score plot (4 LDA components) generated using the prototype OMB model building software using the mass range m/z 600-950 and a bin of 0.05 Da. Clear separation can be seen between the beef (red), goat (blue), lamb (orange) and pork (green) burns with all 50% burger burns being situated in between the two species of which the burgers are made up of. Each data point represents an individual burn.

Minced burger composition (%)	Beef detection	Goat detection	Lamb detection	Pork detection	False postive (FP) / False negative (FN)
Beef : Goat (50 : 50)	✓✓✓	✓✓✓	×××	×××	-
Beef : Goat (80 : 20)	✓✓✓	✓✓✓	×××	×××	-
Beef : Goat (90 : 10)	✓✓✓	✓✓✓	×××	×××	-
Beef : Goat (95 : 5)	✓✓✓	×××	×××	×××	FN
Beef : Goat (98 : 2)	✓✓✓	×××	×××	×××	FN
Beef : Goat (99 : 1)	✓✓✓	×××	×××	×××	FN
Beef : Goat (99.9 : 0.1)	✓✓✓	×××	×××	×××	FN
Beef : Lamb (50 : 50)	✓✓✓	×××	✓✓✓	×××	-
Beef : Lamb (80 : 20)	✓✓✓	×××	✓✓✓	×××	-
Beef : Lamb (90 : 10)	✓✓✓	×××	×××	×××	FN
Beef : Lamb (95 : 5)	✓✓✓	×××	×××	×××	FN
Beef : Lamb (98 : 2)	✓✓✓	×××	×××	×××	FN
Beef : Lamb (99 : 1)	✓✓✓	×××	×××	×××	FN
Beef : Lamb (99.9 : 0.1)	✓✓✓	×××	×××	×××	FN
Beef : Pork (50 : 50)	✓✓✓	×××	×××	✓✓✓	-
Beef : Pork (80 : 20)	✓✓✓	×××	×××	✓✓✓	-
Beef : Pork (90 : 10)	✓✓✓	×××	×××	✓××	-
Beef : Pork (95 : 5)	✓✓✓	×××	×××	×××	FN
Beef : Pork (98 : 2)	✓✓✓	×××	×××	×××	FN
Beef : Pork (99 : 1)	✓✓✓	×××	×××	×××	FN
Beef : Pork (99.9 : 0.1)	✓✓✓	×××	×××	×××	FN
Beef : Goat : Lamb (33 : 33: 33)	✓✓✓	✓✓✓	✓✓✓	×××	-
Beef : Goat : Pork (33 : 33: 33)	✓✓✓	✓✓✓	×××	✓✓✓	-
Beef : Lamb : Pork (33 : 33: 33)	✓✓✓	××✓	✓✓✓	✓✓✓	FP
Beef : Goat : Lamb : Pork (25 : 25: 25: 25)	✓✓✓	✓✓✓	✓✓✓	✓✓✓	-

Table 4.2. Results from adulterated beef burgers with goat, lamb and pork at levels of 50, 20, 10, 5, 2, 1 and 0.1% adulteration made through a serial dilution process and burgers made individually at adulteration levels of 33 and 25%. Species identification of each burn was obtained near-instantaneously using the prototype recognition software. Goat was detected at (10%), lamb (20%) and pork (10-20%). Only one false positive was identified when goat was identified in a beef burger adulterated with lamb and pork.

4.7 References

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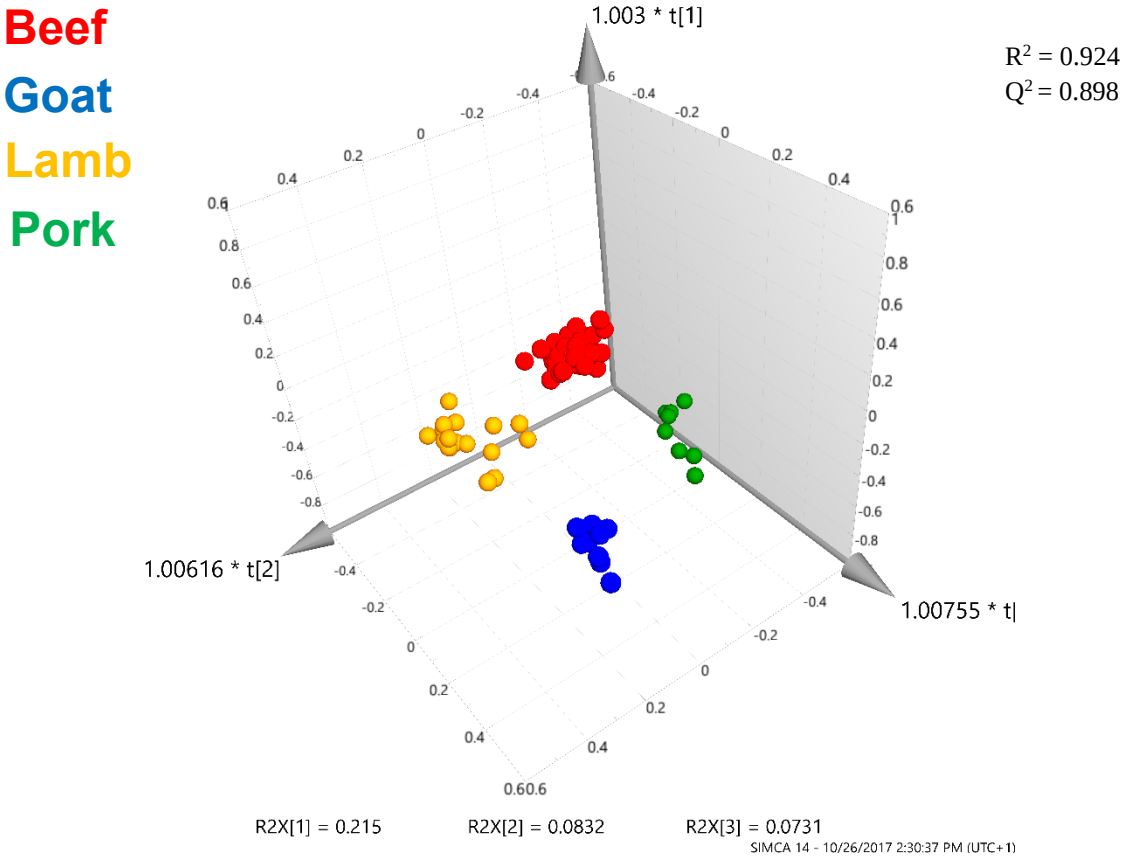
4.8 Appendix

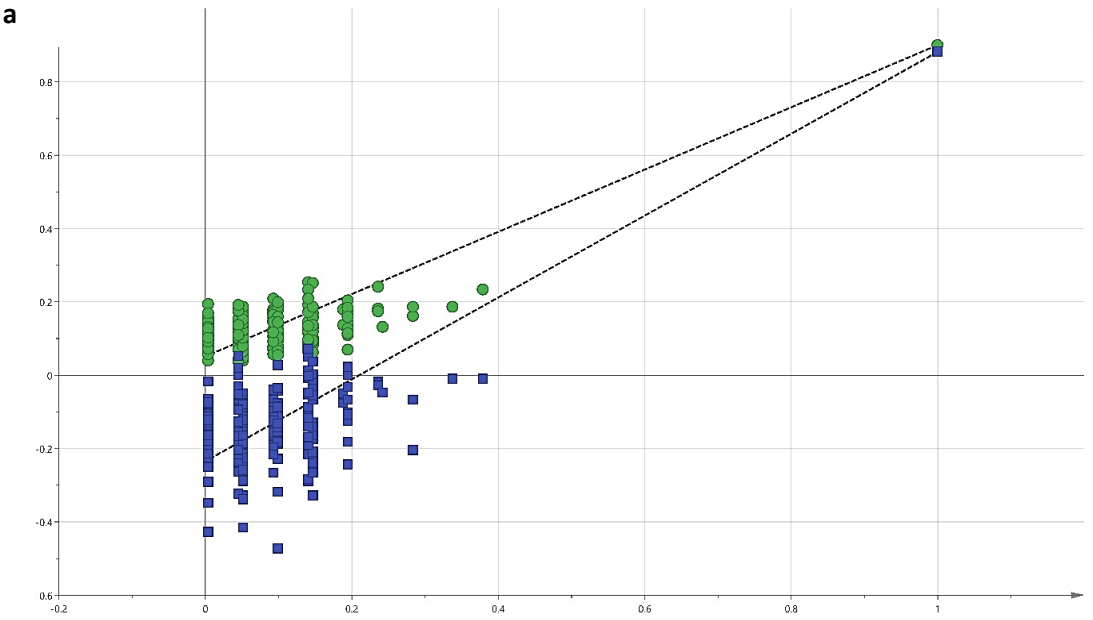
	Beef	Goat	Lamb	Pork	Total	Correct classification rate (%)
Beef	50	0	0	0	50	100.00
Goat	0	10	0	0	10	100.00
Lamb	0	0	18	0	18	100.00
Pork	0	0	0	8	8	100.00
Total					86	100.00

S1 - Results from the leave-20%-out cross-validation of the PCA-LDA speciation models generated using the prototype software in which an overall correct classification rate of 100.00% was achieved.



S2. Orthogonal partial least squares-discriminant analysis (OPLS-DA) (3 latent and 3 orthogonal components) score plot generated using the SIMCA 14. A mass range of m/z 600-950 and bin of 0.05 Da were used to generate the model. Clear separation can be seen between the beef (red), goat (blue), lamb (yellow) and pork (green) samples in which each data point represents one sample.

a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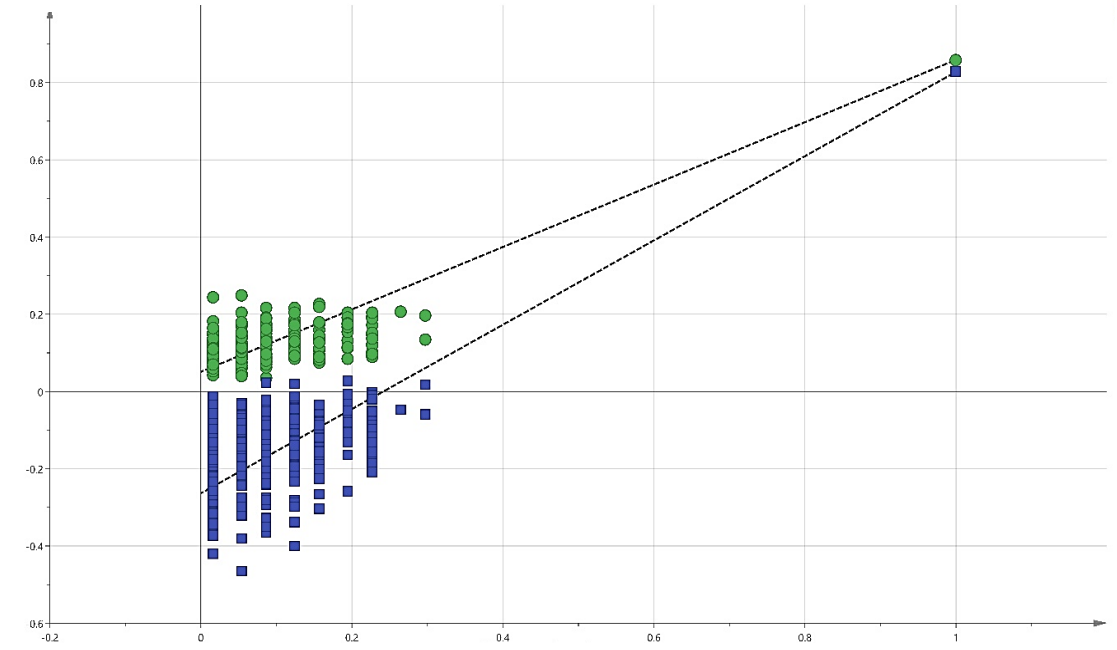
b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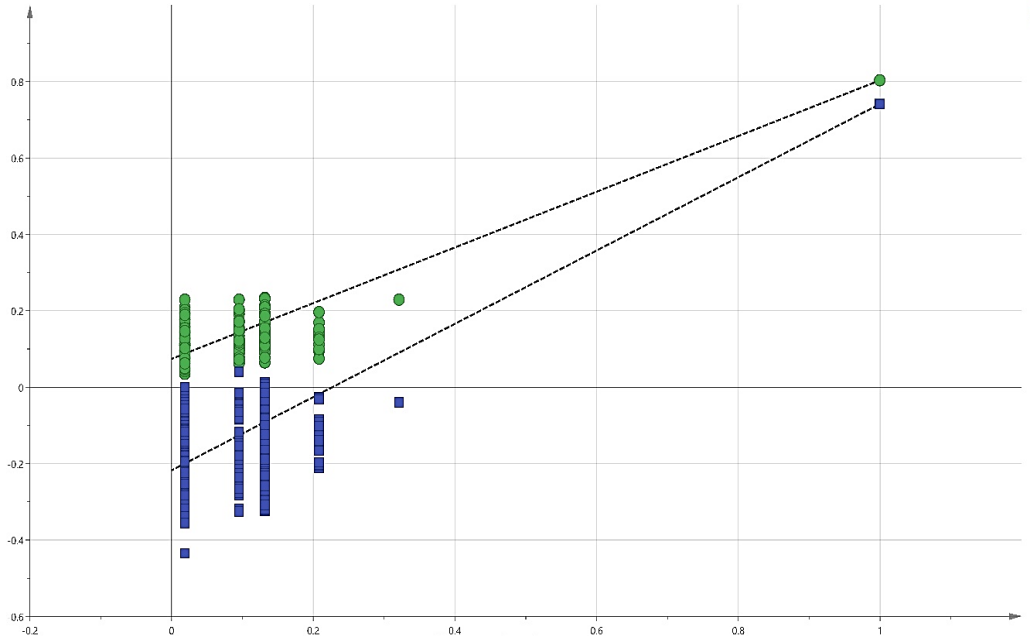
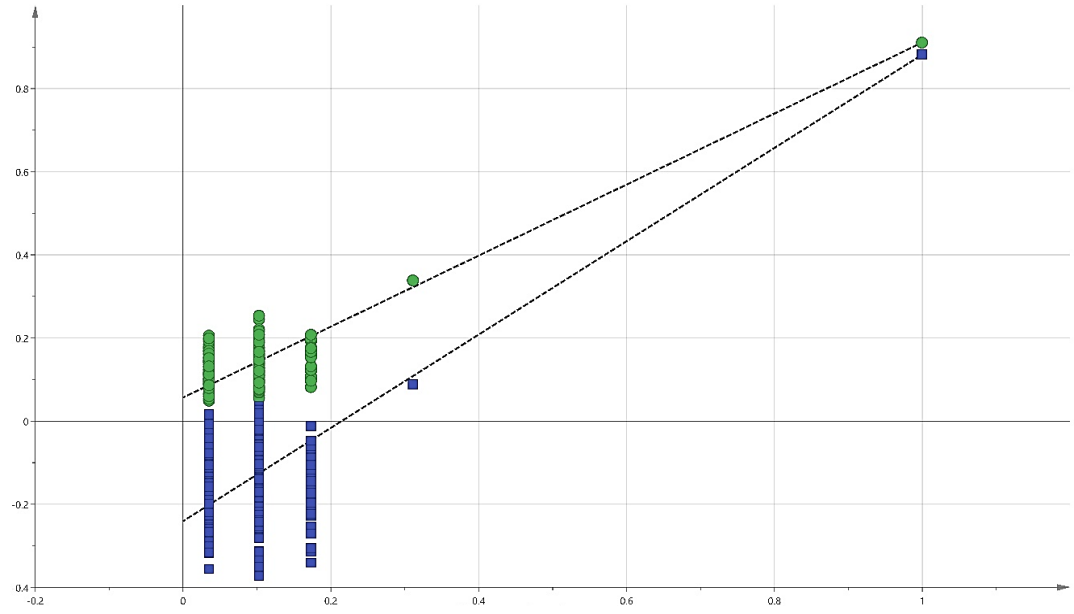
c



d



S3- Permutation tests for each species of meat within the OPLS-DA model (S3); (a) beef; (b) goat; (c) lamb and (d) pork. All tests were carried out using 200 permutations with each Q^2 regression line (blue) intercepting the y-axis beneath the origin suggesting that the OPLS-DA model was not over-fitted.

OPLS-DA model	Latent component	Orthogonal component	R^2 (cum)	Q^2 (cum)	RMSECV
Beef v other species	1	2	0.928	0.912	0.146
Goat v other species	1	4	0.891	0.830	0.132
Lamb v other species	1	3	0.915	0.881	0.140
Pork v other species	1	3	0.938	0.898	0.093

S4- Values of the statistical parameters obtained for the different OPLS-DA models of each species of meat against the other three species under investigation generated using REIMS in negative ionisation mode to identify ions of significance. R^2 (cumulative), Q^2 (cumulative) and Root Mean Squared Error of cross validation (RMSECV) were used to determine the validity of the models. R^2 (cum) indicates the variation described by all components in the model and Q^2 is a measure of how accurately the model can predict class membership.

5. Thesis conclusions and future perspectives

Chapter 5

Food fraud is a deliberate act perpetrated by criminals for additional and substantial economic profits. Most cases involve the adulteration of products ranging from levels of 20-100%. Consumers are unaware, economically cheated and their health is seldom if ever considered by the fraudster. Recent high-profile scandals have bought the fraudulent sale, authenticity, traceability and safety of food to the forefront of the global media. Consumers, authorities and the reputable food industry are now demanding greater controls on the quality, safety, authenticity and traceability of food. Various analytical techniques have been employed to detect food fraud and/or prove the traceability of commodities and ingredients. However, most require long and often complex sample preparation procedures prior to analysis. In terms of preventing fraud in global and fast-moving supply chains this is a substantial disadvantage. The main issue this thesis aimed to address was whether mass spectrometric platforms, especially those which can be operated under ambient conditions, i.e. rapid evaporative ionisation mass spectrometry (REIMS), combined with chemometric analysis can be utilised to detect the fraudulent sale of food. The main findings of each thesis experimental chapter can be summarised as follows:

1. Herbs and spices are commodities which are much more accessible nowadays. This has led to an increase in demand and thus, an increase in price making them very susceptible to fraud. Complex supply chains for herbs and spices is also a major factor. The price of spices is dictated by the intensity of their vibrant colours, making them prone to the addition of dyes. Herbs on the other hand are not traded on colour and instead their price is associated with how compact the product is. This makes them susceptible to the addition of so called bulking agents. The analysis of certified oregano and adulterant samples (olive leaves, myrtle leaves, sumac leaves, cistus leaves and hazelnut leaves) using a two-tier approach of Fourier-transform infrared (FT-IR) and liquid chromatography-high resolution mass spectrometry (LC-HRMS) combined with multivariate analysis identified clear signs of commercial oregano fraud. Fifty-three samples obtained from retail and service sector outlets within the UK/Ireland showed that approximately 24.5% had been

adulterated. Olive and myrtle leaves were found to be the most commonly used adulterants with levels of adulteration ranging from 30% to over 70%. Similar findings were reported for the twenty-five samples analysed from commercial outlets outside the UK/Ireland and internet sites. Differences in the spectroscopic and spectrometric profiles of the oregano and adulterant samples were evident in the principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) plots and further analysis of the spectrometric data allowed several potential unique biomarkers for each adulterant to be identified in both positive (n=16) and negative (n=12) ionisation mode.

2. The substitution of one white fish species for another is common practice and similar to that of oregano adulteration, is close to impossible for consumers and retailers to identify. Whilst genomic profiling studies aimed at fish species identification provide excellent qualitative and quantitative results, this comes at the expense of long and often complex sample procedures. The analysis of five genetically similar white fish species (cod, coley, haddock, pollock and whiting) using REIMS and a prototype recognition software demonstrated that accurate, reliable and near-instantaneous speciation results was achievable. Exploiting the raw spectrometric data to chemometric analysis allowed PCA and (linear discriminant analysis (LDA) models to be generated. Using these as a reference point, raw data from a sample unknown to the models can be acquired, assessed and assigned a fish species classification within seconds. Techniques such as polymerase chain reaction (PCR), which is commonly used for such studies is unable to compete with the speed of the results obtained through REIMS analysis. This was demonstrated by the six suspect samples which had been labelled as ‘haddock’. Both REIMS and PCR analysis correctly concluded that the six samples had been mislabelled and that they were in fact cod. However, time comparisons of fifteen minutes (REIMS) – twenty-four hours (PCR), including all necessary sample preparation to obtain results was witnessed for the six samples. For fisheries hoping to undertake their own quality control (QC) checks, REIMS could be a solution for species identification with large numbers of samples being analysed over a short time frame. However, with REIMS being a costly lab based

technique at present, it will be some time before the technology can be operated in field situations. Analysis of the raw data did not result in the identification of any unique species-specific markers. However, using variable importance in projection (VIP) (x >1), S-plot |p| (x > 0.03) and S-plot |p(corr)| (x > 0.5) parameters, it was possible to identify ions that occurred at predominately higher levels in certain species of fish compared to others. MS/MS analysis identified fragments for each ion. However, based on a database search it was not possible to provide an identification for each ion. Multiple identifications were assigned due to the lack of chromatographic separation that is associated with REIMS analysis. Two phospholipid species; phosphatidylethanolamine (PE) and phosphatidylserine (PS) were assigned with the only exception being m/z 655.5 $[2M-H]^-$ which is believed to be a dimer of docosahexaenoic acid (DHA) (m/z 327.21 $[M-H]^-$).

As well as speciation analysis, separation of two different catch methods (line and trawl caught) of haddock samples was observed. This is the first time that the catch method of fish samples has been scientifically reported. Different catch methods of a fish can result in significant price differences with line caught fish gathering a higher premium than that of trawl caught. Unlike the speciation analysis, it is unclear what types of ions are responsible for the separation within the chemometric models. It is very unlikely that different catch methods would affect the lipidome of a fish sample unless differing diets occurs. A more plausible explanation would be that the stress experienced by a fish would differ however, it is still unclear as to whether the REIMS technology can detect such compounds.

3. Whereas non-processed meat products are susceptible to fraud in terms of geographic origin, sex and breed, processed meats are also prone to the substitution of one species for a cheaper alternative. Species identification was demonstrated successfully when analysing the five different white fish species. However, that study only exhibited the qualitative screening applications of the REIMS technology as a fish fillet can only be 0 or 100% adulterated. The analysis of adulterated beef burgers with three other meat species (goat, lamb and pork) identified that the presence of goat was detectable at levels

as low as 2%, pork at 5% and lamb at 10% using the REIMS technology. Additionally, the identification of multiple meat species at levels ranging from 25-33% were detectable. However, it is evident that preparing burgers through a serial dilution process impacts the quantitative abilities of the REIMS technology with limits of detection (LOD) for each adulterant being higher compared to those not made through serial dilution; goat (10%), lamb (20%) and pork (10-20%). Whilst this is a potential issue which may hinder REIMS future studies, the LOD that were established from both burger making processes are more than sufficient when put into context with the levels that are often found in most commercial meat species substitution cases. The use of chemometrics did not result in the identification of unique species-specific markers. However, ions found to occur more prominently in certain species were based on their VIP ($x > 1$) , S-plot $|p|$ value ($x > 0.03$) and S-plot $|p(corr)|$($x > 0.5$) values. MS/MS fragmentation enabled the assignment of the phospholipid ions which were found to be phosphatidic acid (PA), phosphatidylcholine (PC), PE, phosphatidylinositol (PI) and PS. Due to the lack of chromatographic separation, a mixture of isomeric and isobaric species were assigned to each ion.

The findings within this thesis have addressed the original question which was whether mass spectrometric platforms coupled with chemometric modelling had a prominent role to play in detecting food fraud. However, the results ascertained within this thesis can be extrapolated further and therefore, there are several recommendations as stated below.

The adulterant markers identified in the oregano untargeted MS approach have recently been transferred to a targeted LC-MS/MS method using a triple quadrupole (TQD) mass spectrometer in which total analysis time including sample preparation is approximately thirty minutes.[1] The precursor ions found to produce three significant fragment ions (figure 5.1), yielding 5.5 identification points as defined in Commission Decision 2002/657/EC were selected for the multiple reaction monitoring (MRM) window.[2] It was found that each adulterant contained at least one specific ion. Method validation of the LC-MS/MS method was also based on the Commission Decision 2002/657/EC.[2]

Selectivity and specificity were assessed by analysing multiple oregano and adulterant samples as well as herbs which had previously not been analysed by high resolution mass spectrometry (HRMS), such as sage, to assess potential interferences. Linearity, biodiversity, matrix effects and within laboratory reproducibility (WLR) were also analysed with the latter two involving the analysis of 3 levels of adulteration with 3 mixtures containing both olive and myrtle leaves. Mixture 1 consisted of 10% and 60%; mixture 2– 30% and 30%; mixture 3– 60% and 10% (w/w) of olive and myrtle leaves respectively.[1] The presence of an adulterant at 60% heavily affected the signal of the 10% adulterant with substantial ion enhancement being witnessed for olive leaves (≈ 40%) and ion suppression for myrtle leaves (≈ 30%).

Adulterant	Biomarker	Molecular Ion [m/z]	Fragment Ions [m/z]	CE [V]	MRM window	ESI
Olive leaves	O1-	403.3	100.6	20	7	-
			119.0	20		
			223.2	20		
	O2- *	827.4	487.1 *	40	10	-
			529.6	40		
			781.0	20		
Myrtle leaves	M1 *	555.24	153.2 *	30	4	+
			223.0	20		
			315.0	20		
	M1-	380.9	218.2	40	6	-
			261.0	20		
			291.0	20		
Sumac	S2	1075.0	153.0	40	5	+
			305.0	30		
			457.0	20		
	S3 *	539.0	377.0 *	50	3	+
			403.0	40		
			419.0	20		
Hazelnut leaves	H1	297.2	107.0	20	2	+
			133.0	20		
			191.1	10		
	H2 *	297.1	133.0 *	20	1	+
			177.0	10		
			191.0	10		
	H1-	475.3	118.0	20	9	-
			188.9	30		
			295.0	10		
Phlomis	Ph2- *	450.8	142.7	30	8	-
			224.8 *	20		
			243.0	20		

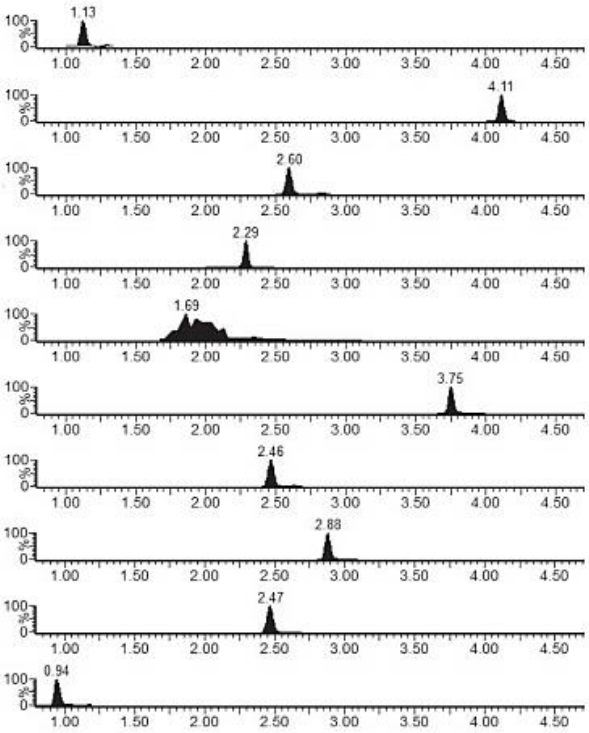


Figure 5.1 Monitored transitions of the selected adulterant markers during targeted LC-MS/MS analysis with the retrospective chromatographic traces (presented in the respective row).[1]

WLR analysis enabled the decision limit (CCα) and detection capabilities (CCβ) of each adulterant to be determined. CCα values of 1.27% and 1.18% and CCβ values of 2.16% and 2.02% were obtained for olive and myrtle leaves respectively.[1] Following method validation, the analysis of fifty-four suspect oregano samples revealed that almost 90% were adulterated with a median level of 50% adulteration.

Moving forward from these findings there are two aspects to consider; (i) whilst the targeted LC-MS/MS method produces results much faster than that of studies which employ untargeted MS or other conventionally used techniques, thirty minutes is still a longer process compared to that of most AMS techniques. It may be possible to transfer this method to a targeted AMS study which would speed up both sample preparation and analysis time. Therefore, oregano samples should be investigated using techniques such as DART and DESI. Perhaps ASAP and REIMS could also be utilised but as stated in chapter 1, there are concerns with regards to the quantitative abilities of ASAP compared to that of DART, DAPCI, LTP and PS. REIMS may suffer a similar fate but a more pressing issue for the technology is that it is difficult to analyse dried products using an electrosurgical knife. However, this could be circumvented using a different probe such as bi-polar forceps or an IR laser. The interaction of the two alternative probes with the herb samples are very likely to differ thus potentially producing different spectral profiles. Therefore, both would need to be assessed. A two-tier system employing fast spectroscopic profiling and near-instantaneous spectrometric analysis would be extremely beneficial to the herb and spice industry and indeed many other industries. (ii) Secondly, and arguably a more pressing aspect to consider for the herb and spice industry is that if substantial amounts of adulteration are being identified in oregano samples, then it is likely that similar amounts of adulteration are being witnessed in other popular herbs such as sage, thyme and parsley. Therefore, analysis of these herbs and potential adulterants needs to be carried out to establish if they too are being exposed to fraud and if so to what extent.

The results obtained from the fish speciation results are encouraging and it is evident that the technology could enable fisheries and processors to conduct their own QC checks, albeit REIMS is expensive and non-field deployable at present. An issue which is apparent, not only

in the REIMS fish study but also the other two studies conducted within this thesis is the lack of confirmative identifications that have been assigned to each ion. Although the selection of the ions has been statistically justified as mentioned throughout each chapter, a confirmatory identification is much more difficult due to the large number of isobaric and isomeric isomers that can be associated for each ion. With regards to the adulterant markers identified within the oregano study, the lack of confirmative identifications is not as much of an issue as the LC-MS/MS study has shown that a handful of markers are unique and the acquisition of 5.5 identification points, as well as retention time fulfil the minimum criteria for characterisation of unknown metabolites outlined by Chemical Analysis Working Group within Metabolomics Standards Initiative.[1,3] With regards to the ions that been identified in both the fish and meat studies, those are not unique to any species. Therefore, there are several steps that can be undertaken to assign confirmative identifications. Firstly, the majority of putative identifications assigned are available as commercial standards. Therefore, it should be possible to assess the fragmentation pattern of the standards within the REIMS source by adding them to isopropanol (IPA) which is continuously infused to aid the ionisation process. Comparison with the MS/MS fragmentations of the selected ions within the fish and meat samples would potentially clarify the presence of certain lipid species. Secondly, an attempt should be made to analyse the samples using LC-HRMS and perhaps another AMS technique. With regards to LC-HRMS analysis, chromatographic separation may clarify what lipid species are present. Various lipidomic extraction methods should be studied, most notably the Folch,[4] Bligh-Dyler[5] and more recent Matyash method.[6] The latter approach appears to be very popular within studies at present because it utilises methyl-tert-butyl ether (MTBE) allowing the lipid rich organic layer to sit above the denser aqueous layer as opposed to the other two approaches. Both reversed-phase liquid chromatography (RPLC), which separates compounds on their hydrophobicity (lipophilicity) and hydrophilic interaction chromatography (HILIC) which separates compounds based on their hydrophilicity should be employed. The combination of the two strategies would provide the best avenue for a confirmative identification, although ion suppression may still be an issue due to the presence

of isobaric and isomeric species. Alternatively, the coupling of the REIMS source with ion mobility spectrometry (IMS) may provide better conformational measurements as it rapidly separates ions with the same exact mass based on their shape and mobility, otherwise known as drift time Additionally, the acquisition of spectrometric data in continuum mode using the MS^E function (as conducted in the oregano study) may improve biomarker identification/classification.

Thermal imprinting–easy ambient sonic spray ionisation-mass spectrometry (TI-EASI-MS) is an AMS technique that requires very little sample preparation and has been used to analyse both meat (beef, chicken, mutton and pork) and fish (salmon, sardine and trout) samples.[7] The literature suggests that it is possible to acquire spectra that are dominated by lipids and therefore, it may be possible to carry out a comparison with the REIMS technology. Free fatty acids (FFA) and triacyclglycerols (TAG) have been used as the main point of discrimination and the application of chemometrics has identified separation between the samples. Several FFA have also been identified within the REIMS studies undertaken within this thesis and therefore, attempts should be made to assess the comparisons between the two AMS techniques. With EASI being a soft ionisation technique, very little fragmentation will occur allowing intact ions to be analysed.

As stated in this thesis there are six other ‘sins’ in which fish fraud can manifest itself. Although we have demonstrated that catch method is an aspect of fish fraud which maybe distinguishable, a much larger study aimed at multiple fish species, especially those which have such a gradient in pricing (tuna, seabass, salmon) is required. Additionally, other aspects of fish fraud such as geographic origin and farming methods need to be addressed as it is very likely that these are also often exploited by fraudsters. Like meat products, processed and cooked fish samples will be most prone to fraud. However, it is very unlikely that the raw fish speciation model generated in this thesis can be used to analyse cooked samples due to potential changes in the phospholipid composition. Thus, a model based solely on cooked samples will need to be generated. Within that, the various cooking methods that are associated with fish products (frying, boiling, steaming etc.) will need to be considered as it

is very likely that the profiles of the different cooking methods will vary. Likewise, a model of cooked meat samples should be generated to determine the capabilities of the REIMS technology. The addition of seasoning products, including herbs and spices may also need to be considered as they could impact the quality of the results and species classification. There are early indications from a previous REIMS meat study that both cooking, and the addition of seasoning products do not interfere with species separation when applying chemometrics.[8] However, as we have established there are concerns that heavily processed or vigorously mixed samples impact the quantitative abilities of the REIMS technology. But, there is always the possibility that homogenisation of the samples was not suitable when preparing (blending) the adulterated beef burgers. The addition of dyes to adulterant samples could be a possibility in future studies to ensure that sufficient homogenisation occurs. Additionally, the use of different probes such as bipolar forceps and IR lasers should be assessed to see what impact they have on the spectral profile of the samples. Perhaps bipolar forceps are not best suited for this application as only small amounts of sample are analysable at any given time. To detect adulteration of processed meat and fish products, as many data and sampling points as possible are required. Additionally, the cleaning up procedure for the bipolar forceps is most likely to be more time consuming than that of an electrosurgical knife.

Moving forward there are a few aspects of the REIMS technology to consider. Automation is common within not only most mass spectrometer instruments but several analytical platforms allowing multiple samples to be analysed over lengthy periods without the need for an analyst to be present at all times. Currently REIMS requires each sample to be individually analysed by an operative user. An auto sampler would circumvent this issue and allow more time to be spent analysing the data. Different types of probes should be analysed ranging in size and shape to assess what importance that has on the quality of acquired raw data. Finally, as mentioned throughout this thesis, the development of miniature and fieldable mass spectrometers needs to move at a more rapid space so that techniques such as REIMS can be implemented into commercial and retail outlets so that companies can undertake their own QC checks.

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